From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 November 2000 (02.11.00)	
International application No. PCT/US00/06456	**Applicant's or agent's file reference**
International filing date (day/month/year) 10 March 2000 (10.03.00)	**Priority date** (day/month/year) 11 March 1999 (11.03.99)
Applicant KAEPPLER, Shawn, M. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

10 October 2000 (10.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was [ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Pascal Piriou Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 00/06456	International filing date *(day/month/year)* 10/03/2000	(Earliest) Priority Date *(day/month/year)* 11/03/1999
Applicant WISCONSIN ALUMNI RESEARCH FOUNDATION et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

CLASS II DNA METHYLTRANSFERASES OF ZEA MAYS

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ________

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

Inte[illegible]onal Application No
P[illegible]S 00/06456

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/10 C12N15/63 C12N5/14 C12N15/83 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Olhoft P.M.: "Cloning and characterization of the 5-methylcytosine methyltransferase gene in maize (zea mays) plants and tissue cultures" UNIV. OF MINNESOTA (0130) Degree: PHD Date:1998 pp:137 ABSTR. INT. B 1999, 59 (9),4638;Avail.: UMI,Order No. DA9907518 XP000900933	1-33
A	-& OLHOFT P.M. ET AL.: "Zea mays DNA (cytosine-5)-methyltransferase gene, complete sequence" EMBL DATABASE ENTRY T01661; ACCESSION NO. T01661, 19 February 1999 (1999-02-19), XP002146224	1-33

	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2000	25/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schönwasser, D

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	CAO X. ET AL.: "Conserved plant genes with similarity to mammalian de novo DNA methyltransferase" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 97, no. 9, 25 April 2000 (2000-04-25), page 4979-4984 XP002146225 figure 3	1-33
A	HENIKOFF S. ET AL.: "A DNA methyltransferase homolog with a chromodomain exists in multiple polymorphic forms in Arabidopsis" GENETICS, vol. 149, no. 1, May 1998 (1998-05), pages 307-318, XP002146226 the whole document	1-18
P,A	WALBOT V.: "Maize ESTs from various cDNA libraries sequences at Stanford University; 687002G02.y1 687 - Early embryo from Delaware Zea mays cDNA, mRNA sequence." EMBL DATABASE ENTRY AW065905; ACCESSION NO.:AW065905, 18 October 1999 (1999-10-18), XP002146227	1-18
P,A	WALBOT V.: "Maize ESTs from various cDNA libraries sequences at Stanford University;707027A05.x2 707 - Mixed adult tissues from Walbot lab (SK) Zea mays cDNA, mRNA sequence." EMBL DATABASE ENTRY AW330561; ACCESSION NO. AW330561, 1 February 2000 (2000-02-01), XP002146228	1,19-33

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PATENT COOPERATION TREATY

RECD 25 MAY 2001
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
WIS49870051PCT		
International application No. PCT/US00/06456	International filing date (day/month/year) 10/03/2000	Priority date (day/month/year) 11/03/1999
International Patent Classification (IPC) or national classification and IPC C12N9/10		
Applicant WISCONSIN ALUMNI RESEARCH FOUNDATION et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☒ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☒ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
10/10/2000	22.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Roscoe, R Telephone No. +49 89 2399 2554

Form PCT/IPEA/409 (cover sheet) (January 1994)

I. Basis of th r p rt

1. With regard to the **l ments** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-57 as originally filed

Claims, No.:

1-21 as received on 16/03/2001 with letter of 16/03/2001

Drawings, sheets:

1/39-39/39 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:
   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-21
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-21
Industrial applicability (IA)	Yes:	Claims	1-21
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

II. Priority

Since present application is based on specific sequences, the question whether these sequences are present in the priority documents is the primary determinant for priority. The first priority document does not define any of the present sequences. Said document discloses a polypeptide which matches present Fig.2A from position 93-900 but which has a different C-terminal sequence. Hence, none of the present claims are considered entitled to the first priority date (11.03.99). The second priority document (09.12.99) establishes priority for the protein sequence depicted in present Fig.2A (Fig.2 of prio), and for the DNA sequence depicted in present Fig.1A (Fig.1 of prio). Hence, claims 2 and 3 are entitled to the second priority date. The other claims to zmet2a can become entitled to this date if the enzyme is defined on the basis of the sequences of Fig.1A and 2A and do not contain other matter which is not entitled to priority. It seems that all embodiments listed would be entitled to priority from (09.12.99) if linked to priority-entitled sequence definition.

If an incomplete sequence is provided in a priority document, the complete sequence cannot be entitled to priority from said document. This is not a question of enablement. The skilled person could simply not know what the actual sequence of the complete gene is by looking at the priority document. Hence, although a methyltransferase comprising the partial sequence could be entitled to priority, the complete sequence defined as such could not.

IV. Lack of Unity

zmet2a and zmet2b are considered non-unitary since they are mere further examples of methyltransferase genes. This objection shall however not be persued in the International Phase. However, should applicant wish to enter a European Regional Phase, he is requested to file seperate applications for each of the two genes.

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

The documents mentioned in the present International Preliminary Examination

Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc. (presently have documents D1-D6)

Applicants contribution to the art is as follows. Applicant took an EST with similarity to known methyltransferases (EST corresponded to 151-2569 of Fig1A or 1B). The EST was enlarged by standard techniques to provide a complete reading frame. Further, plants comprising a zmet2a::Mu1 mutant allele were used to show that reduced methylation took place as a result of the mutation. zmet2b is simply a clone from a maize genomic library which hybridized to a zmet2a probe - no functional data is provided, neither is a complete sequence of the gene.

- **Novelty (Art.33(2) PCT)**

D1 discloses Zmet1 methyltransferase of Zea mays. It further suggests the existence of a second Zmet1 loci in the plants studied. The sequence of D1 shows 29.4% identity to zmet2a in 677aa overlap. The sequence is not cited against the present claims.

D4 relates to multiple polymorphs of a DNA methyltransferase in Arabidopsis. The protein has 49.9% identity in a 786 aa overlap. No significant DNA identity levels appear to have been detected, hence not cited against present claims.

In view of the priority situation, D5 is relevant to all of the present claims. D5 discloses a Zea mays cDNA sequence of undefined function which has 98% identity in a 499 bp overlap with zmet2a. This sequence will undoubtedly hybridize to the genes of the application but is unlikely to encode a functional methyltransferase.

D6 discloses a Zea mays cDNA sequence of undefined function which has 78.5% identity in a 489 bp overlap with zmet2b. D6 is relevant to claims relating to zmet2b, since these are not entitled to priority until the filing date. Expected to hybridize to genes of present invention, yet unlikely to encode functional methyltransferase.

- **Inv ntiv St p (Art.33(3) PCT)**

No inventive activity can be detected in the present application. The problem solved by the applicant is to find further plant methyltransferase genes (or in case of zmet2b to find further methyltransferase-like sequences). The solutions are the sequences of the invention. Posed with the problem of finding plant methyltransferases, a skilled person will look for characteristic methyltransferase sequences in an EST database (at both DNA and 6 reading frame translated levels) or will perform hybridization screening. Both of these approaches could have been used to isolate the genes of the present invention. In the former computer-based approach, a skilled person would take the prior art methyltransferases and (i) directly search for similar sequences or (ii) look for conserved sequences and search for these in a database. The suggestion in D1 that another methyltransferase exists in Zea mays is invitation enough to look for this enzyme. A skilled person could use sequence information from D1 or D2 as a basis for such a search and would have expected to be successful because most classes of enzymes retain characteristic motifs. Applicant would have to credibly explain why a search would not be expected to be successful in the present case before inventive step could be acknowledged.

- **Industrial Applicability (Art.33(4) PCT)**

The present claims appear to have industrial applicability.

VIII. **Certain observations**

- **Clarity (Art.6 PCT)**

Claims 1, 3, 13 - define "stringent conditions"

PATENT COOPERATION TREATY

LVM
WIS4987P0051PC

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: MUELLER, Lisa V. ROCKEY, MILNAMOW & KATZ, LTD 180 North Stetson Avenue 2 Prudential Plaza Suite 4700 Chicago, IL 60601 ETATS-UNIS D'AMERIQUE	**PCT** NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT (PCT Rule 71.1)
	Date of mailing *(day/month/year)* 22.05.2001

RECEIVED
MAY 30 2001
Rockey, Milnamow & Katz, Ltd.

Applicant's or agent's file reference WIS49870051PCT		**IMPORTANT NOTIFICATION**
International application No. PCT/US00/06456	International filing date *(day/month/year)* 10/03/2000	Priority date *(day/month/year)* 11/03/1999
Applicant WISCONSIN ALUMNI RESEARCH FOUNDATION et al.		

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

   The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

   Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

   For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/ European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer CLEERE, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

WHAT IS CLAIMED IS:

1. An isolated and purified DNA sequence which encodes a *Zea mays* zmet2a methyltransferase and which hybridizes to the nucleic acid sequence shown in FIG. 1A under stringent conditions.

2. An isolated and purified zmet2a methyltransferase comprising the amino acid sequence shown in FIG. 2A.

3. An isolated and purified DNA sequence which encodes a *Zea mays* zmet2b methyltransferase and which hybridizes to the nucleic acid sequence shown in FIG. 1B under stringent conditions.

4. An isolated and purified zmet2a methyltransferase comprising the amino acid sequence shown in FIG. 2B.

5. A recombinant expression cassette comprising the isolated and purified nucleic acid sequence of claims 1 or 3, a promoter sequence and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the nucleic acid sequence and the nucleic acid sequence is operably linked to the polyadenylation signal sequence.

6. The recombinant expression cassette of claim 5 wherein the promoter sequence is a constitutive or a tissue specific promoter sequence.

7. A bacterial cell comprising the recombinant expression cassette of claim 5.

8. The bacterial cell of claim 7 wherein the bacterial cell is selected from the group consisting of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

9. A transgenic plant comprising the recombinant expression cassette of claim 5.

10. The transgenic plant of claim 9 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

11. The transgenic plant of claim 10 wherein transgenic plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

12. Seed comprising the recombinant expression cassette of claim 5.

13. An isolated and purified DNA sequence which encodes a *Zea mays* zmet2b methyltransferase and which hybridizes to the nucleic acid sequence of FIG. 23 under stringent conditions.

14. A recombinant expression cassette comprising the isolated and purified nucleic acid sequence of claim 13, a promoter sequence and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the nucleic acid sequence and the nucleic acid sequence is operably linked to the polyadenylation signal sequence.

15. The recombinant expression cassette of claim 14 wherein the promoter sequence is a constitutive or a tissue specific promoter sequence.

16. A bacterial cell comprising the recombinant expression cassette of claim 14.

17. The bacterial cell of claim 16 wherein the bacterial cell is selected from the group consisting of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

18. A transgenic plant comprising the recombinant expression cassette of claim 14.

19. The transgenic plant of claim 18 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

20. The transgenic plant of claim 19 wherein transgenic plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

21. Seed comprising the recombinant expression cassette of claim 14.

WHAT IS CLAIMED IS:

1. An isolated and purified *Zea mays* zmet2a methyltransferase nucleic acid sequence.

2. The nucleic acid sequence of claim 1 wherein the nucleic acid sequence hybridizes to the nucleic acid sequence of FIG. 1A under stringent conditions.

3. A zmet2a methyltransferase comprising the amino acid sequence shown in FIG. 2A.

4. The nucleic acid sequence of claim 1 wherein the nucleic acid sequence hybridizes to the nucleic acid sequenceof FIG. 1B under stringent conditions.

5. A zmet2a methyltransferase comprising the amino acid sequence shown in FIG. 2B.

6. A recombinant expression cassette comprising the isolated and purified nucleic acid sequence of claim 1, a promoter sequence and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the nucleic acid sequence and the nucleic acid sequence is operably linked to the polyadenylation signal sequence.

7. The recombinant expression cassette of claim 6 wherein the promoter sequence is a constitutive or a tissue specific promoter sequence.

8. A recombinant expression cassette comprising a heterologous nucleic acid sequence, a promoter sequence from the nucleic acid sequence of claim 1 and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the heterologous nucleic acid sequence and the heterologous nucleic acid sequence is operably linked to the polyadenylation signal sequence.

9. A bacterial cell comprising the recombinant expression cassette of claims 6 or 8.

10. The bacterial cell of claim 9 wherein the bacterial cell is selected from the group consisting of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

11. A transgenic plant cell comprising the recombinant expression cassette of claims 6 or 8.

12. The transgenic plant cell of claim 11 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

13. A transgenic plant comprising the recombinant expression cassette of claims 6 or 8.

14. The transgenic plant of claim 13 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

15. The transgenic plant of claim 13 wherein transgenic plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

16. Seed from the transgenic plant of claim 13.

17. A process for methylating a target gene in a plant, the process comprising the steps of:

transforming a plant with a recombinant expression cassette comprising a tissue specific promoter and the nucleic acid sequence of claim 1, the tissue specific promoter being operably linked to the nucleic acid sequence, wherein the tissue-specific promoter directs expression of the nucleic acid sequence, and the expression of the nucleic acid

sequence produces zmet2a methyltransferase in sufficient quantities in the area containing the target gene to allow for methylation of the target gene.

18. The process of claim 17 wherein the plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

19. An isolated and purified *Zea mays* zmet2b methyltransferase nucleic acid sequence.

20. An isolated and purified *Zea mays* zmet2b methyltransferase nucleic acid sequence which hybridizes to FIG. 23 under stringent conditions.

21. A recombinant expression cassette comprising the isolated and purified nucleic acid sequence of claim 19, a promoter sequence and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the nucleic acid sequence and the nucleic acid sequence is operably linked to the polyadenylation signal sequence.

22. The recombinant expression cassette of claim 21 wherein the promoter sequence is a constitutive or a tissue specific promoter sequence.

23. A recombinant expression cassette comprising a heterologous nucleic acid sequence, a promoter sequence from the nucleic acid sequence of claim 19 and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the heterologous nucleic acid sequence and the heterologous nucleic acid sequence is operably linked to the polyadenylation signal sequence.

24. A bacterial cell comprising the recombinant expression cassette of claims 21 or 23.

25. The bacterial cell of claim 24 wherein the bacterial cell is selected from the group consisting of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

26. A transgenic plant cell comprising the recombinant expression cassette of claims 21 or 23.

27. The transgenic plant cell of claim 26 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

28. A transgenic plant comprising the recombinant expression cassette of claims 21 or 23.

29. The transgenic plant of claim 28 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

30. The transgenic plant of claim 28 wherein transgenic plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

31. Seed from the transgenic plant of claim 28.

32. A process for methylating a target gene in a plant, the process comprising the steps of:

transforming a plant with a recombinant expression cassette comprising a tissue specific promoter and the nucleic acid sequence of claim 19, the tissue specific promoter being operably linked to the nucleic acid sequence, wherein the tissue-specific promoter directs expression of the nucleic acid sequence, and the expression of the nucleic acid sequence produces zmet2b methyltransferase in sufficient quantities in the area containing the target gene to allow for methylation of the target gene.

33. The process of claim 32 wherein the plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

LVM

PATENT COOPERATION TREATY

File Amendments 11/25/00

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:

ROCKEY, MILNAMOW & KATZ, LTD
2 Prudential Plaza, Suite 4700
Attn. MUELLER, L.
180 North Stetson Avenue
Chicago, Ill. 60601
UNITED STATES OF AMERICA

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

RECEIVED
SEP 26 2000
Rockey, Milnamow & Katz, Ltd

	Date of mailing (day/month/year) 25/09/2000
Applicant's or agent's file reference WIS4987P0051PC	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 00/06456	International filing date (day/month/year) 10/03/2000
Applicant WISCONSIN ALUMNI RESEARCH FOUNDATION et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Renate Jordan

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

WIS4987P0051PC
LVM

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

MUELLER, Lisa V.
ROCKEY, MILNAMOW & KATZ, LTD.
180 North Stetson Avenue
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Suite 4700
Chicago, IL 60601
ETATS-UNIS D'AMERIQUE

RECEIVED
DEC 27 2000
Rockey, Milnamow & Katz, Ltd.

PCT

Reply to Written opinion 3/19/01

WRITTEN OPINION

(PCT Rule 66)

Date of mailing (day/month/year)	19.12.2000	
Applicant's or agent's file reference WIS49870051PCT	**REPLY DUE**	**within 3 month(s)** from the above date of mailing
International application No. PCT/US00/06456	International filing date (day/month/year) 10/03/2000	Priority date (day/month/year) 11/03/1999
International Patent Classification (IPC) or both national classification and IPC C12N9/10		
Applicant WISCONSIN ALUMNI RESEARCH FOUNDATION et al.		

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain document cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

   When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

   How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

   Also: For an additional opportunity to submit amendments, see Rule 66.4.
   For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
   For an informal communication with the examiner, see Rule 66.6.

   If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 11/07/2001.

Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer / Examiner Roscoe, R
	Formalities officer (incl. extension of time limits) Emslander, S Telephone No. +49 89 2399 8718

Form PCT/IPEA/408 (cover sheet) (January 1994)

I. Basis of the opini n

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".)*:

Description, pages:

1-57 as originally filed

Claims, No.:

1-33 as originally filed

Drawings, sheets:

1/39-39/39 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

IV. Lack of unity of invention

1. In response to the invitation (Form PCT/IPEA/405) to restrict or pay additional fees, the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied with for the following reasons and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees:
   see separate sheet

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

   ☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrialapplicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1, 2, 4, 19, 20
Inventive step (IS)	Claims	1-33
Industrial applicability (IA)	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
se separate sheet

I. Basis

The documents mentioned in the present written opinion / International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc. (presently have documents D1-D6)

II. Priority

Since present application is based on specific sequences, the question whether these sequences are present in the priority documents is the primary determinant for priority. The first priority document does not define any of the present sequences. Said document discloses a polypeptide which matches present Fig.2A from position 93-900 but which has a different C-terminal sequence. Hence, none of the present claims are considered entitled to the first priority date (11.03.99). The second priority document (09.12.99) establishes priority for the protein sequence depicted in present Fig.2A (Fig.2 of prio), and for the DNA sequence depicted in present Fig.1A (Fig.1 of prio). Hence, claims 2 and 3 are entitled to the second priority date. The other claims to zmet2a can become entitled to this date if the enzyme is defined on the basis of the sequences of Fig.1A and 2A and do not contain other matter which is not entitled to priority. It seems that all embodiments listed would be entitled to priority from (09.12.99) if linked to priority-entitled sequence definition.

IV. Lack of Unity

zmet2a and zmet2b are considered non-unitary since they are mere further examples of methyltransferase genes. This objection shall however not be persued in the International Phase. *However, should applicant wish to enter a European Regional Phase, he is requested to file seperate applications for each of the two genes.*

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

Applicants contribution to the art is as follows. Applicant took an EST with

similarity to known methyltransferases (EST corresponded to 151-2569 of Fig1A or 1B). The EST was enlarged by standard techniques to provide a complete reading frame. Further, plants comprising a zmet2a::Mu1 mutant allele were used to show that reduced methylation took place as a result of the mutation. zmet2b is simply a clone from a maize genomic library which hybridized to a zmet2a probe - no functional data is provided, neither is a complete sequence of the gene.

- **Novelty (Art.33(2) PCT)**

D1 discloses Zmet1 methyltransferase of Zea mays. It further suggests the existence of a second Zmet1 loci in the plants studied. The sequence of D1 shows 29.4% identity to zmet2a in 677aa overlap. The sequence is not cited against the present claims.

D4 relates to multiple polymorphs of a DNA methyltransferase in Arabidopsis. The protein has 49.9% identity in a 786 aa overlap. No significant DNA identity levels appear to have been detected, hence not cited against present claims.

In view of the priority situation, D5 is relevant to all of the present claims. D5 discloses a Zea mays cDNA sequence of undefined function which has 98% identity in a 499 bp overlap with zmet2a. This sequence will undoubtedly hybridize to the genes of the application and is thus cited against claims 1, 2, 4, 19 and 20

D6 discloses a Zea mays cDNA sequence of undefined function which has 78.5% identity in a 489 bp overlap with zmet2b. D6 is relevant to claims relating to zmet2b, since these are not entitled to priority until the filing date. Expected to hybridize to genes of present invention - thus cited against claims 1, 2, 4, 19 and 20.

- **Inventive Step (Art.33(3) PCT)**

No inventive activity can be detected in the present application. The problem solved by the applicant is to find further plant methyltransferase genes (or in case of zmet2b to find further methyltransferase-like sequences). The solutions are the

sequences of the invention. Posed with the problem of finding plant methyltransferases, a skilled person will look for characteristic methyltransferase sequences in an EST database or will perform hybridization screening. Both of these approaches could have been used to isolate the genes of the present invention.

- **Industrial Applicability (Art.33(4) PCT)**

The present claims appear to have industrial applicability.

VIII. **Certain observations**

- **Clarity (Art.6 PCT)**

Claim 1 - "Zea mays zmet2a" is an arbitrary definition and defines at best the problem applicants set out to solve. Claim needs to comprise solution i.e. sequence information.

Claims 2, 4, 20 - define "stringent conditions"

Claims 11 and 13 - identical (same applies to claims 26 and 28)

Claim 16, 31 - transgene must be in germ-line i.e. better to define that seed itself has transgene.

Claim 20 - hybridizes to <u>sequence of</u> Fig.

- **Support in description (Art.6, PCT)**

Claims 17, 18, 32, 33 - Applicant does not demonstrate how to target methylation to a specific target gene. Hence, these claims are not supported (the skilled person is also not enabled to perform this method).

Claims 8 and 23 and further claims insofar as dependent thereon - applicant has not isolated a functional promoter of either zmet2a or zmet2b. Promoter isolation

often proves difficult, especially if one wants to obtain a promoter-containing fragment of a size amenable for use in a vector for expressing heterologous genes.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 September 2000 (14.09.2000)

PCT

(10) International Publication Number
WO 00/53732 A3

(51) International Patent Classification[7]: C12N 9/10, 15/63, 5/14, 15/83, 15/82

(21) International Application Number: PCT/US00/06456

(22) International Filing Date: 10 March 2000 (10.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/123,888 11 March 1999 (11.03.1999) US
60/169,858 9 December 1999 (09.12.1999) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/169,858 (CIP)
Filed on 9 December 1999 (09.12.1999)

(71) Applicants *(for all designated States except US)*: **WISCONSIN ALUMNI RESEARCH FOUNDATION** [US/US]; P.O. Box 7365, Madison, WI 53707-7365 (US). **PIONEER HI-BRED, INTERNATIONAL, INC.** [US/US]; Suite 800, 400 Locust Street, P.O. Box 800, Des Moines, IA 50306-3453 (US). **REGENTS OF THE UNIVERSITY OF MINNESOTA** [US/US]; 600 University Gate Way, 200 Oak Street S.E., Minneapolis, MN 55455 (US).

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(74) Agents: **MUELLER, Lisa, V.** et al.; Rockey, Milnamow & Katz, Ltd., Suite 4700, Two Prudential Plaza, 180 North Stetson Avenue, Chicago, IL 60601 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report: 21 December 2000

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/53732 A3

(54) Title: CLASS II DNA METHYLTRANSFERASES OF ZEA MAYS

(57) Abstract: The present invention provides nucleic acids encoding polypeptides which encode a DNA methyltransferase. These nucleic acids can be used to stabilize transgene expression in transgenic plants, to alter the yield or biochemical qualities of plants to silencing targeted genes in plants *in vivo*.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 9/10, 15/63, 5/14, 15/83, 15/82 — A2

(11) International Publication Number: WO 00/53732

(43) International Publication Date: 14 September 2000 (14.09.00)

(21) International Application Number: PCT/US00/06456

(22) International Filing Date: 10 March 2000 (10.03.00)

(30) Priority Data:
60/123,888 11 March 1999 (11.03.99) US
60/169,858 9 December 1999 (09.12.99) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/169,858 (CIP)
Filed on 9 December 1999 (09.12.99)

(71) Applicants *(for all designated States except US)*: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; P.O. Box 7365, Madison, WI 53707-7365 (US). PIONEER HI-BRED, INTERNATIONAL, INC. [US/US]; Suite 800, 400 Locust Street, P.O. Box 800, Des Moines, IA 50306-3453 (US). REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; 600 University Gate Way, 200 Oak Street S.E., Minneapolis, MN 55455 (US).

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(74) Agents: MUELLER, Lisa, V. et al.; Rockey, Milnamow & Katz, Ltd., Suite 4700, Two Prudential Plaza, 180 North Stetson Avenue, Chicago, IL 60601 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: CLASS II DNA METHYLTRANSFERASES OF ZEA MAYS

(57) Abstract

The present invention provides nucleic acids encoding polypeptides which encode a DNA methyltransferase. These nucleic acids can be used to stabilize transgene expression in transgenic plants, to alter the yield or biochemical qualities of plants to silencing targeted genes in plants *in vivo*.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

CLASS II DNA METHYLTRANSFERASES OF ZEA MAYS

FIELD OF THE INVENTION

The present invention relates to nucleic acid and amino acid sequences which encode class II DNA methyltransferases. The present invention further relates to methods of using the nucleic acid and amino acid sequences described herein to stabilize transgene expression in transgenic plants, to alter the yield or biochemical qualities of plants and to silence targeted genes in plants *in vivo*.

BACKGROUND OF THE INVENTION

The information content of a primary DNA sequence can be enhanced by the addition of a methyl group to the ring structure of cytosine or adenine residues (Finnegan, E.J., et al., *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:223-47 (1998)). The chemical modification of DNA is known to affect protein-DNA interactions. Specifically, in prokaryotes, methylation of DNA prevents cleavage by the cognate restriction endonucleases. *Id.* In higher eukaryotes, cytosine methylation can inhibit binding of regulatory proteins and methylation of promoter and coding sequences of genes can repress transcription, both *in vitro* and *in vivo*. *Id.* Methylation of DNA has been implicated in the timing of DNA replication, in determination of chromatin structure, in increasing mutation frequency, as a causal agent for some human diseases, and as a basis for epigenetic phenomena. *Id.*

Eukaryotic genomes are not methylated uniformly, but instead contain specific methylated regions, with other domains remaining unmethylated (Martienssen, R.A., et al., *Current Opinion in Genetics and Development*, 5:234-242 (1995)). The enzymes that transfer methyl groups to the cytosine ring are cytosine-5-methyltransferases (hereinafter referred to as "DNA methyltransferases") and have been characterized from a number of eukaroytes. All characterized eukaryotic DNA methyltransferases exhibit little primary sequence specificity *in vitro* other than the short canonical symmetrical sites methylated which are CpG in animals, and CpG and CpNpG in plants (where N stands for any nucleotide). Mammalian and plant

genomes contain methylation-free GC-rich zones, or CpG islands, which are frequently associated with the 5' regions of housekeeping genes. *Id.*

In plants, DNA methylation is necessary for normal development. For example, Arabidopsis having reduced levels of DNA methylation demonstrate a range of abnormalities, including loss of apical dominance, reduced stature, altered leaf size and shape, reduced root length, homeotic transformation of floral organs and reduced fertility (Finnegan, E.J., et al., *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:223-47 (1998)). Moreover, Arabidopsis plants in which methylation had been reduced by at least 70% became infertile after four to five generations of selfing. *Id.* A comparable reduction in DNA methylation is embryo lethal in mammals. *Id.*

Two classes of DNA methyltransferase enzymes have been cloned in plants (Finnegan, E.J., et al., *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:223-47 (1998)) - class I and class II. Class I enzymes include MetI and MetII from Arabidopsis (Finnegan et al. *Nucleic Acids Res.*, 21(10):2383-2388 (1993); Nebendahl, et al., *Gene* 157(1-2):269-272 (1995)), Met1-5 and Met2-21 from carrot (Bernacchia, G et al., *Plant Physiol.* 116:446-446 (1998)), C-5 MTase from tomato (Bernacchia, G et al. *Plant J.*, 13(3):317-330 (1998)), and C-5 MTase from pea (Pradhan et al., *Nucleic Acids Res.*, 26(5):1214-1222 (1998)). Class II sequences have been detected in many species with a defining characteristic of the presence of an embedded chromodomain (Rose et al., *Nucleic Acids Res.*, 26(7):1628-1635 (1998)). The only full-length class II sequence is Cmt1 from Arabidopsis (Genbank #AF039364).

Class I enzymes are homologous to dnmt1 from mice (Bestor, T., et al., *EMBO J.*, 11(7):2611-2617 (1988)), the first cloned DNA methyltransferase. A knockout of dnmt1 in mice resulted in lethality during embryogenesis (Li et al., *Cell*, 69(6):915-926 (1992)). Dnmt1 has been used as a model for all class I enzymes though it has not been proven whether this is appropriate in plant systems. Antisense expression of MetI in Arabidopsis resulted in numerous developmental abnormalities (Finnegan et al., *Proc. Natl. Acad. Sci. U.S.A.*, 93(16):8449-8454 (1996)). Class I enzymes are thought to function as maintenance enzymes, though proteolytic cleavage could create de novo enzymes (Bestor, T.H., *EMBO J.*, 11(7):2611-2617

(1992)). CpG activity has been shown for dnmt1 in mice and humans. In peas it was found that pea C-5 MTase expressed in baculovirus displayed both CpG and CpNpG activity (Pradhan et al., *Nucleic Acids Res.*, 26(5):1214-1222 (1998)). In general, class I enzymes have a high level of expression in tissues that are actively dividing and are expressed at lower levels or silent in mature tissues.

There is little known regarding the function of class II enzymes. CmtI was detected as an Arabidopsis genomic sequence based on sequence homology to other methyltransferases. The C-terminal region contains the conserved methyltransferase domains and a chromodomain. The N-terminal region is much shorter than the N-terminal region of class I enzymes. Several commonly used ecotypes of Arabidopsis contain an allele of Cmt1 which is interrupted by a transposon insertion. These Cmt1 knockouts do not have any detectable phenotype. No other research has been published on the function of class II enzymes. Cmt1 is expressed only in floral tissues at very low levels. Degenerate PCR has been used to show the presence of Cmt1 homologs in a number of other plant species (Rose et al., *Nucleic Acids Res.*, 26(7):1628-1635 (1998)). In addition to finding homologs in other species, two sequences with similarity to Cmt1, Cmt2 and Cmt3, were identified in the Arabidopsis.

DNA methylation provides a mechanism for the mitotic propagation of epigenetic states. Epigenetic lineage-dependent patterns of gene expression have been studied the most in the germline and in somatic cell lineages in multicellular eukaryotes (Martienssen, R.A., et al., *Curr. Opin. Genet. and Develop.*, 5:234-242 (1995)). For example, in mice, the parentally imprinted genes *H19* and *Igf2r* are expressed in the embryo only when they are inherited via the female gamete. *Id.* In contrast, the *Igf2* gene is expressed only when inherited via the male gamete. *Id.* The human homologs of the *Igf2* and *H19* genes are linked and parentally imprinted as in the mouse. *Id.* Parental uniparental disomy for this chromosomal region (11p15) is associated with Beckwith-Wiedemann syndrome, which is believed to result from overexpression of *Igf2*. *Id.* In addition to overgrowth of certain organs, Beckwith-Wiedemann syndrome patients have a 700-fold predisposition to Wilms' tumor, and loss of heterozygosity in this region is found in many other tumors as well. *Id.* It has

also been shown that 60-70% of Wilms' tumor patients have biallelic expression of *Igf2*, *H19*, or both in tumor tissue, resulting from loss of imprinting rather than loss of heterozygosity. *Id.*

In plants, epigenetic changes in gene expression are considered to be easier to observe than in animals since there is little cell migration and clonal lineages stay together. *Id.* Moreover, because in plants the germline arises relatively late in development, many somatically variegated phenotypes can be followed into the next generation and are heritable to greater or lesser extents. *Id.* Parental imprinting of gene expression was first observed in plants at the *R* locus in maize. *Id.* Certain alleles condition a mottled phenotype in the alerone layer of the extra-embryonic endosperm when inherited paternally, but cause a fully colored phenotype when inherited maternally. *Id.* Genetic studies of modifier loci have revealed that it is the maternally inherited *R* allele that is imprinted to a high level of expression. *Id.* High levels of R expression correlate with demethylation of sites in the transcribed region in the maternally inherited allele. *Id.*

Plants transformed with additional copies of endogenous genes or with multiple copies of a foreign or exogenous gene (these endogenous and exogenous genes are often referred to as "transgenes") frequently display epigenetic inactivation. This phenomenon is known as "gene silencing" or "co-suppression". There are two types of "gene silencing" or "co-suppression". The first is "transcriptional silencing". In "transcriptional silencing", RNA production from the introduced transgene is repressed. The second type of "gene silencing" is "posttranscriptional silencing". In "posttranscriptional silencing", transcripts do not accumulate in the cytoplasm even though transcription rates are comparable with or are higher than those in cells where transcripts do accumulate.

Transcriptional silencing is associated with transgene methylation, particularly in the promoter (Finnegan, E.J., et al., *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:223-47 (1998)). Posttranscriptional silencing, which affects both transgenes and homologous endogeneous genes, is also associated with transgene methylation, but within the coding sequence rather than the promoter. *Id.* It is believed that both

forms of gene silencing reflect normal, cellular defenses against invading or mobile DNAs. *Id.*

Currently, two classes of methyltransferase genes have been cloned in maize. The class I clone homolog is referred to as Zmet1 and the class II homolog Zmet2. The Zmet1 is a class I enzyme that was cloned by Paula Olhoft and Ron Phillips at the University of Minnesota. FIG. 4 is a summary of the major classes of 5-cytosine methyltransferases from mammals, *Arabidposis* and maize. The present invention herein relates to zmet2a and zmet2b methyltransferases.

SUMMARY OF THE INVENTION

In one embodiment, the present invention relates to an isolated and purified *Zea mays* zmet2a methyltransferase nucleic acid sequence. Specifically, the isolated and purified *Zea mays* zmet2a methyltransferase nucleic acid sequence of the present invention hybridizes to the nucleic acid sequences shown in FIG. 1A and 1B under stringent conditions. The zmet2a methyltransferase nucleic acid sequence encodes the enzyme zmet2a methyltransferase. The amino acid sequences for zmet2a methyltransferase is shown in FIG. 2A and FIG. 2B.

In another embodiment, the present invention further relates to recombinant expression cassettes comprising the isolated and purified zmet2a nucleic acid sequence described herein. Preferably, the recombinant expression cassettes further contain a promoter sequence and a polyadenylation signal sequence. The promoter sequence can be operably linked to the zmet2a nucleic acid sequence. The zmet2a nucleic acid sequence is operably linked to the polyadenylation signal sequence. Any promoter sequence can be used in the recombinant expression cassette, such as, but not limited to a constitutive or tissue specific promoter.

In another embodiment, the present invention also relates to a recombinant expression cassettes comprising one or more heterologous nucleic acid sequences. Such recombinant expression cassettes further contain a promoter sequence from the zmet2a nucleic acid sequence and a polyadenylation signal sequence. The promoter sequence is operably linked to the heterologous nucleic acid sequence. The

heterologous nucleic acid sequence is operably linked to the polyadenylation signal sequence. Any heterologous promoter sequence can be used in this recombinant expression cassette.

In a further embodiment, the present invention also relates to bacterial cells comprising at least one of the recombinant expression cassettes described herein. The bacterial cells can be *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*.

In a further embodiment, the present invention further relates to transgenic plant cells and transgenic plants containing the recombinant expression cassettes described herein. Monocotyledonous or dicotyledonous plant cells and plants can be transformed with the hereinbefore described recombinant expression cassettes. Plants which can be transformed with the recombinant expression cassettes of the present invention include, but are not limited to, *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, *Brassica napus*, etc. The present invention also relates to seed resulting from the transgenic plants of the present invention.

In a further embodiment, the present invention further provides methods of reducing or altering methyltransferase activity in a transgenic plant in order to increase transgene expression stability and/or to improve the yield or biochemical qualities of a plant as well as a method of silencing targeted genes in a plant *in vivo*. These methods comprise introducing into a plant a recombinant expression cassette comprising an appropriate plant promoter operably linked to a zmet2a methyltransferase nucleic acid sequence described herein in either the sense or antisense direction.

In a further embodiment, the present invention relates to an isolated and purified *Zea mays* zmet2b methyltransferase nucleic acid sequence. The zmet2b methyltransferase nucleic acid sequence of the present invention can be isolated using an isolated and purified partial *Zea mays* zmet2b methyltransferase nucleic acid sequence. The isolated and purified partial *Zea mays* zmeb2b methyltransferase

nucleic acid sequence can be used as a probe to isolate the zmet2b methyltransferase nucleic acid encoding zmet2b methyltransferase. Preferably, the isolated and purified partial *Zea mays* zmet2b methyltransferase nucleic acid described herein hybridizes to FIG. 23 under stringent conditions. The partial zmet2b methyltransferase nucleic acid sequence described herein encodes a portion of zmet2b methyltransferase. The partial amino acid sequence of zmet2b methyltransferase is shown in FIG. 24. The zmet2b methyltransferase nucleic acid sequence can be used in recombinant expression cassettes in the same manner as the isolated and purified zmet2a nucleic acid sequence described herein. Such recombinant expression cassettes can be used to create transgenic plants containing these recombinant expression cassettes. Additionally, the zmet2b methyltransferase nucleic acid sequence can be used to reduce or alter methyltransferase activity in transgenic plants in the same manner as the zmet2a methyltransferase nucleic acid sequence.

Definitions

Units, prefixes, and symbols can be denoted in the SI accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation, respectively. The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny thereof. The class of plants which can be used in the methods of the present invention are generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

As used herein, "heterologous" when used to describe nucleic acids or polypeptides refers to nucleic acids or polypeptides that originate from a foreign species, or, if from the same species, are substantially modified from their original form. For example, a promoter operably linked to a heterologous structural gene is

from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form.

A nucleic acid or polypeptide is "exogenous to" an individual plant is one which is introduced into the plant by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation, biolistic methods, electroporation, and the like. Such a plant containing the exogenous nucleic acid is referred to herein as an R_1 generation transgenic plant. Transgenic plants which arise from sexual cross or by selfing are descendants of such a plant.

As used herein, "zmet2a methyltransferase gene" or "zmet2a methyltransferase nucleic acid" refers to a nucleic acid encoding zmet2a methyltransferase and which hybridizes under stringent conditions and/or has at least 60% sequence identity at the deduced amino acid level to the exemplified sequences provided herein. The zmet2a polypeptide encoded by the zmet2a methyltransferase gene has at least 55% or 60% sequence identity, typically at least 65% sequence identity, preferably at least 70% sequence identity, often at least 75% sequence identity, more preferably at least 80% sequence identity, and most preferably at least 90% sequence identity at the deduced amino acid level relative to the exemplary zmet2a methyltransferase sequences provided herein.

As used herein, "zmet2a methyltransferase nucleic acid" includes reference to a contiguous sequence from a zmet2a methyltransferase gene of at least 2454 nucleotides in length. In some embodiments the nucleic acid is preferably at least 2736 nucleotides in length (see FIG. 1A) and more preferably at least 2796 nucleotides in length (see FIG. 1B).

As used herein, "zmet2b methyltransferase gene" or "zmet2b methyltransferase nucleic acid" refers to a nucleic acid encoding zmet2b methyltransferase and which can be identified using the partial zmet2b methyltransferase nucleic acid shown in FIG. 23. The zmet2b methyltransferase gene

hybridizes under stringent conditions to the partial zmet2b methyltransferase nucleic acid shown in FIG. 23.

As used herein, "a partial zmet2b methyltransferase nucleic acid" includes reference to a contiguous sequence of at least 1181 nucleotides in length and which is from the zmet2b methyltransferase gene.

As used herein, "isolated" includes reference to material which is substantially or essentially free from components which normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence includes the complementary sequence thereof.

As used herein, "operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to joint two protein coding regions, contiguous and in the same reading frame.

In the expression of transgenes, one of ordinary skill in the art will recognize that the inserted nucleic acid sequence need not be identical and may be "substantially identical" to a sequence of the gene from which it was derived. As explained below, these variants are specifically covered by this term.

In the case where the inserted nucleic acid sequence is transcribed and translated to produce a functional zmet2a and/or zmet2b methyltransferase

polypeptide, one of ordinary skill in the art will recognize that because of codon degeneracy, a number of nucleic acid sequences will encode the same polypeptide. These variants are specifically covered by the term "zmet2a methyltransferase nucleic acid sequence" or "zmet2b methyltransferase nucleic acid sequence". In addition, the term specifically includes those full length sequences substantially identical (determined as described below) with a zmet2a and/or zmet 2b methyltransferase gene sequence which encode proteins that retain the function of the zmet2a and/or zmet2b methyltransferase. Thus, in the case of the zmet2a and/or zmet2b methyltransferase genes described herein, the term includes variant nucleic acid sequences which have substantial identity with the sequences disclosed herein and which encode proteins capable of reducing or regulating DNA methylation in a transgenic plant for various purposes as well as silencing target genes in a plant using the nucleic acid sequences described herein.

Two nucleic acids or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The term "complementary to" is used herein to mean that the complementary sequence is identical to all or a specified contiguous portion of a reference nucleic acid sequence. Sequence comparisons between two (or more) nucleic acids or polypeptides are typically performed by comparing sequences of two optimally aligned sequences over a segment or "comparison window" to identify and compare local regions of sequence similarity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Ad. App. Math.* 2: 482 (1981), by the homology alignment algorithm of Neddleman and Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* (U.S.A.) 85:2444 (1988), by computerized implementation of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (hereinafter "GCG"), 575 Science Dr., Madison, WI), or by inspection.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, where the portion of the nucleic acid

sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity" of nucleic acid sequences means that a nucleic acid comprises a sequence that has at least 55% or 60% sequence identity, generally at least 65%, preferably at least 70%, often at least 75%, more preferably at least 80% and most preferably at least 90%, compared to a reference sequence using the programs described above (preferably BESTFIT) using standard parameters. One of ordinary skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid sequences for those purposes normally means sequence identity of at least 55% or 60%, preferably at least 70%, more preferably at least 80%, and most preferably at least 95%. Polypeptides having "sequence similarity" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

Another indication that nucleic acid sequences are substantially identical is if two molecules hybridize to each other under appropriate conditions. Appropriate

conditions can be high or low stringency and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C to about 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH 0) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically. stringent wash conditions are those in which the salt concentration is about 0.22 molar at pH 7 and the temperature is at least about 50°C. However, nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

Nucleic acids of the present invention can be identified from a cDNA or genomic library prepared according to standard procedures and the nucleic acids disclosed here used as a probe. For example, stringent hybridization conditions will typically include at least one low stringency wash using 0.3 molar salt (e.g., 2X SSC) at 65°C. The washes are preferably followed by one or more subsequent washes using 0.03 molar salt (e.g., 0.2X SSC) at 50°C, usually 60°C, or more usually 65°C. Nucleic acid probes used to isolate the nucleic acids are preferably at least 100 nucleotides in length.

As used herein, a homologue of a particular zmet2a and/or zmet2b methyltransferase gene is a second gene (either in the same species or in a different species) which encodes a protein having an amino acid sequence having at least 50% identity or 75% similarity to (determined as described above) to a polypeptide sequence in the first gene product.

As used herein, "nucleotide binding site" or "nucleotide binding domain" includes reference to a region consisting of kinase-la, kinase 2, and kinase 3a motifs, which participates in ATP/GTP-binding. Such motifs are described for instance in Yu *et al., Proc. Acad. Sci. USA* 93:11751-11756 (1996); Mindrinos. *et al., Cell* 78:1089-1099 and Shen *et al.*, FEBS, 335:380-385 (1993).

As used herein. "tissue-specific promoter" includes reference to a promoter in which expression of an operably linked gene is limited to a particular tissue or tissues.

As used herein "recombinant" includes reference to a cell. or nucleic acid, or vector. that has been modified by the introduction of a heterologous nucleic acid or the alteration of a native nucleic acid to a form not native to that cell. or that the cell is derived from a cell so modified. For example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

As used herein. a "recombinant expression cassette" is a nucleic acid construct. generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid in a target cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of the expression vector includes a nucleic acid to be transcribed. and a promoter..

As used herein, "transgenic plant" includes reference to a plant modified by introduction of a heterologous nucleic acid. Generally, the heterologous nucleic acid is a zmet2a and/or zmet2b methyltransferase structural or regulatory gene or subsequences or combinations thereof.

As used herein, "hybridization complex" includes reference to a duplex nucleic acid sequence formed by selective hybridization of two single-stranded nucleic acids with each other.

As used herein, "amplified" includes reference to an increase in the molarity of a specified sequence. Amplification methods include the polymerase chain reaction (hereinafter "PCR"), the ligase chain reaction (hereinafter "LCR"), the transcription-based amplification system (hereinafter "TAS"). the self-sustained sequence replication system (hereinafter "SSR"). A wide variety of cloning methods,

host cells. and *in vitro* amplification methodologies are well-known to persons of ordinary skill in the art.

As used herein. "nucleic acid sample" includes reference to a specimen suspected of comprising a zmet2a and/or zmet2b methyltransferase gene.

SEQUENCE LISTINGS

The present application contains a number of nucleotide sequences and amino acid sequences. For the nucleotide sequences, the base pairs are represented by the following base codes:

Symbol	Meaning
A	A: adenine
C	C; cytosine
G	G; guanine
T	T; thymine
U	U; uracil
M	A or C
R	A or G
W	A or T/U
S	C or G

Symbol	Meaning
Y	C or T/U
K	G or T/U
V	A or C or G; not T/U
H	A or C or T/U; not G
D	A or G or T/U; not C
B	C or G or T/U; not A
N	(A or C or G or T/U)

The amino acids shown in the application are in the L-form and are represented by the following amino acid-three letter abbreviations:

Abbreviation	Amino acid name
Ala	L-Alanine
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartic Acid
Asx	L-Aspartic Acid or Asparagine
Cys	L-Cysteine
Glu	L-Glutamic Acid

Gln	L-Glutamine
Glx	L-Glutamine or Glutamic Acid
Gly	L-Glycine
His	L-Histidine
Ile	L-Isoleucine
Leu	L-Leucine
Lys	L-Lysine
Met	L-Methionine
Phe	L-Phenylalanine
Pro	L-Proline
Ser	L-Serine
Thr	L-Threonine
Trp	L-Tryptophan
Tyr	L-Tyrosine
Val	L-Valine
Xaa	L-Unknown or other

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the nucleic acid sequence of the zmet2a methyltransferase gene containing 2736 basepairs. FIG. 1B shows the nucleic acid sequence of the zmet2a methyltransferase gene containing 2796 basepairs.

FIG. 2A shows the amino acid sequence of the zmet2a methyltransferase containing 912 amino acids and which is encoded by the nucleic acid sequence shown in FIG. 1A. FIG. 2B shows the amino acid sequence of the zmet2a methyltransferase containing 932 amino acids and which is encoded by the nucleic acid sequence shown in FIG. 1B.

FIG. 3 shows the PCR primers used to sequence the zmet2a methyltransferase gene.

FIG. 4 is a summary of the major classes of 5-cytosine methyltransferases from mammals, *Arabidopsis* and maize.

FIG. 5 shows the genomic sequence of zmet2a methyltransferase gene and the retrotransposon SPRITE-1, along with intron-exon divisions, a restriction site map and a primer map.

FIG. 6 lists the World Wide Web sites used to process the sequence data for the zmet2a methyltransferase gene.

FIG. 7 shows a Southern blot of B73 DNA digested with *Hind*III and probed with clone CGET064. The Southern blot shows the presence of multiple copies of zmet2a or zmet2a-like genes in the B73 genome. DNA from B73 was digested with HindIII and probed with clone CGET064 which does not contain a HindIII site. The gene cloned and sequenced is represented by the upper band.

FIG. 8 shows the alignment of the amino acid sequence from zmet2a with the amino acid sequence of *Arbadiopsis* chromosmethylase *CMT1* (AF039367) and the C-terminal methylase domains from the DNA methyltransferases of maize zmet1 (AF063403) and *Arabidopsis MET1* (P34881). Zmet2a shows similarity along the entire length of *CMT1* but significant similarity with zmet1 and Met1 exists only in the conserved motifs. Bold, uppercase, normal uppercase letters, and lower case letters indicate identity, conservation, and differences in amino acid sequences relative to zmet2a respectively. Dashes in the sequences are gaps introduced by CLUSTAL W to optimize the alignments. The location of the six conserved methylase motifs are indicated in the sequence. The chromodomain is located upstream and adjacent to motif IV. The *Mu* insertion into the coding region of motif IX alters zmet2a function resulting in decreased methylation at CpNpG sites. Putative nuclear loalization signal peptides, NLS (N. Raikhel, *Plant Physiol.* 100, 1627 (1992)) are positioned in the N-terminal portion of the protein.

FIG. 9 lists the putative identification of zmet2a amino acids involved in catalysis by comparison with amino acids of M.HhaI with known catalytic functions. The amino acids of M.HhaI with catalytic functions were determined by crystalography by Cheng et al., *Cell*, 74:299-307 (1993). Amino acid of zmet2a are numbered as in Figure 7.

FIG. 10 shows southern analysis of repetitive DNA methylation patterns. Total genomic DNA (5 μg per lane) from an F_4 derived F_5 family segregating for

zmet2a:*Mul* was digested with isoschizomers *Hpa*II and *Msp*I which recoginze the sequence CCGG. Digested DNA ws electophoresed through 0.8% agarose, transferred to nylon membrane, and hybridized with probes for repetitive DNA; the 9kb 26s-5.8s-17s ribosomal repeat (FIG. 10A), 5s ribosomal repeat (FIG. 10B), and a centromeric repeat pSau3a9 (FIG. 10C). Decreased methylation is observed in mutant plants (- -) relative to nonmutant plants (+ +) digested with *Msp*I which is sensitive to methylation at ^{me}CpCpG sequnces. No changes in methylation patterns at ^{me}CpG sits are observed in mutant plants as indicated by the lack of digestion with *Hpa*II. Plants heterozygous for zmet2a:*Mul* (+ -) also show decreases at ^{me}CpCpg sites.

FIG. 11 shows gels from a Southern analysis which demonstrate that plants homozygous for zmet2a::Mu1 have decreased methylation at CpNpG sites. More sites cut with restriction enzymes that are sensitive to methylation at CpNpG sites in zmet2a:*Mul* plants. *Eco*RII is sensitive to methylation at CC*A/TGG sites where * indicates the senitive cytosine (FIG. 11A). *Bgl*II is sensitive to methylation at AGATC*T sites (FIG. 11B). *Pst*I is sensitive to methylation at C*TGCAG sites (FIG. 11C). *Bam*HI is sensitive to methylation at GGATC*C sites (FIG. 11D). *Ava*II is sensitive to methylation at GGA/TC*C sites (FIG. 11E). Changes at CpG sites cannot be sparated from CpCpG in the *Ava*II digests. DNA from the same plants as those in Figure 10 were digested and hybridized with the repetitive probes as described herein.

FIG. 12 shows the cytosine methylation levels in an F4 derived F5 segregating line for zmet2a::Mu1. 5-methylcytosine content of DNA extracted from tissue of immature 5^{th} –7^{th} leaves was determined by reverse phase HPLC using the method of Gehrike et al. Values were obtained from three wildtype plants, seven heterozygous plants and five homozygous plants. Two samples were run for each plant. Percentages of 5mC content [5mC/(5mC + C)] were calculated from concentrations determined from integration of peak and comparison to known standards.

FIG. 13 shows gels from a Southern analysis which demonstrate that plants homozygous for zmet2a::Mu1 having a reduced level of methylation that is stable

over generations. Two F_2 derived F_3 families homozygous for zmet2a:Mul. B5 and B6, were self pollinated to the F_6 generation. Two lineages from B5 and three lineages from B4 were grown at the University of Wisconsin. West Madison Agronomy Farm in 1999. Methylation levels are consistent across generations. Once zmet2a:*Mul* is in a homozygous state, methylation is reduced to a specific level and no further reductions occur. Dilution of methylation is not observed in each successive generation. DNA from leaf tissue was digested with *Msp*I and the Southern blot was hybridized with 9kb ribosomal repetitive probe.

FIG. 14 shows gels from a Southern analysis which demonstrate that methylation levels are restored to nonmutant parental levels in backcross progeny homozygous for wildtype zmet2a. An F1 hybrid of an F4 line homozygous for zmet2a::*Mul* (lanes 1-3) and the inbred line Mo17 (lanes 4-6) was backcrossed to the nonmutant Mo17 parent ot generate plants homozygous wildtype and plants heterozygous for zmet2a:*Mul*. F1 plants (lanes 7-11) have methylation levels intermediate those of the parents. BC1 progeny heterozygous for zmet2a:*Mul* (lanes 12-17) have methylation levels similar to the F1. BC1 plants restored to wild-type zmet2a (lanes 18-21) have remethylation to levels comparable to the nonmutant parent line. Complete or near complete remethylation has occurred within one sexual generation. DNA was extracted from the 4^{th} – 6^{th} immature leaves of greenhouse grown seedlings, digested with *Pst*I which is sensitive to methylation at ^{me}CTGCAG sequences, and hybridized to the pSau3a9 centromeric repeat.

FIG. 15 shows gels from a Southern analysis which demonstrate the expression of zmet2a in different tissues during development. Southern blots were produced with cDNA's synthesized from mRNA extracted from embryos 24 days after pollination (hereinafter "DAP"), young leaves, immature ear, immature tassel, BMS callus, and 10 day old seedlings. Figure 15A shows the ethidium bromide stained gel. All lanes were loaded with 750 ng of cDNA except for the 10 day seedlings, of which 280 ng was loaded due to the limited amount available. The cDNA's were quantified by spectrophotometry. The marker lane contains 800 ng of lambda DNA digested with *Hind*III. Figure 15B shows the Southern blot hybridized with the zmet2a cDNA probe. Hybridization is observed in tissues that are actively

undergoing cell division. Figure 15C shows the same blot hybridized to a ubiquitin probe to show cDNA loading variation.

FIG. 16 shows the structure of maize retrotransposon SPRITE-1 and sequence of Long Terminal Repeat (hereinafter "LTR") components. FIG. 16A shows that SPRITE-1 consists of long terminal direct repeats, a tRNA primer binding site (hereinafter "PBS"), coding sequence for proteins necessary for replication and transposition, and a polypurine tract (hereinafter "PPT"). FIG. 16B identifies the sequences for the 5' and 3' LTR , PBS and PPT. Each LTR has a 3 base pair inverted repeat which is also shown in the drawing. A putative TATA box is underlined and the putative transcription start site is italicized. The 5 base pair host insertion site duplications are also identified.

FIG. 17 shows the alignments of the conserved protein motifs of the Ty1/copia elements with SPRITE-1. The maize retrotransposon SPRITE-1 is aligned with the retrotransposon hopscotch (U2626) from maizze, retrofit (U72725) from rice, an unpublished *Arabidopsis* retrotransposon (AC006528) and the copia element from Drosophila (M11240).

FIG. 18 shows that the SPRITE-1 copy number and insertion sites differ among maize inbred lines. DNA (7 μg) from inbred maize lines, barley, ice, rye, wheat, and potato was digested with BcoRI which does not cut within the retroelement sequence. The Southern blot was hybridized with a 950 bp SPRITE-1 fragment which includes the 5' untranslated sequence and 5' sequence putatively coding for the *gag* protein but does not include the conserved *gag* motif or the 5' terminal repeat.

FIG. 19 shows the identification of inbred lines containing a SPRITE-1 insertion in zmet2a. PCR was conducted on maize inbred lines from various origins using a primer upstream of the SPRITE-1 insertion site 15F in conjuction with a SPRITE-1 specific primer 18R or a zmet2a primer downstream of the element 8R. The upper panel (15F/18R) show the inbreds that do not have a SPRITE-1 insertion. The lower panel (15F/18R) shows that Mo17 and A682 have a SPRITE-1 insertion

into zmet2a. A682 has an amplification product from both primer sets indicating that it may be hemizygous for SPRITE-1.

FIG. 20 shows expression of retroelement SPRITE-1. Figure 20A shows a Southern blot of cDNAs from roots, immature embryo 24 days after pollination (hereinafter, "DAP"), young leaf, young leaf with inacive zmet2a immature ear, immature tassel, mature pollen, Black Mexican Sweet (hereinafter, "BMS") callus, and 10 day seedling, hubridized with a SPRITE-1 probe. Transcription of SPRITE-1 is evident as indicated by the hybridization to cDNA from embryo, and leaf tissue. Expression is highest in leaf tissue with significantly more expression being observed in leaf tissue from zmet2a:Mul plants that have decreased CpNpG methylation. FIG. 20B shows the same Southern blot hybridized to a ubiquitin probe as a loading control.

FIG. 21 shows that the presence of a SPRITE-1 insertion into a zmet2a intron does not alter transcript splicing. Fragmetns spanning the SPRITE-1 insertion and downstream from the insertion site were amplified by PCR from cDNA's. FIG. 21A shows a scaled representation of zmet2a. Exons are represented by large blocks while the intervening introns are depicted by lines. The insertion of the retroelement is indicated above the zmet2a diagram. The element is inserted in the opposite orientation relative to zmet2a as indicated by the boxed arrows which represent the direct repeats. Positions of the primers used to generate fragments are indicated below the zmet2a diagram. Fragments were amplified from B73 (FIG. 21B) immature ear cDNA which does not contain the retroelement insertion and Mo17 (M) embryo 24 days after pollination cDNA (FIG. 21B) and Mo17 (M) 10 day seedling cDNA (FIG. 21C). No differences were observed on the ethidium bromide stained gel of the PCR products. FIGS. 21B and 21C show hybridization of a near full length B73 cDNA probe to a Southern blot of the PCR fragments.

FIG. 22 shows the methylation status of SPRITE-1. DNA from immature leaves was digested with methylation sensitive restriction enzymes. Southern blots were hybridized with a 970 base pair fragment from the 5' end of the untranslated region of SPRITE-1. There are 5 BstNI/EcoRII sites, 1 MspI/HpaII sites and 1 PstI

site within the sequence context of this probe. Nearly all sites are methylated in this region.

FIG. 23 shows a partial nucleic acid sequence of the zmet2b methyltransferase gene.

FIG. 24 shows a partial amino acid sequence of the zmet2b methyltransferase encoded by the partial nucleic acid sequence shown in FIG. 23.

FIG. 25 shows a comparison of a portion of the amino acid sequence for zmet2a methyltransferase with a portion of the amino acid sequence for zmet2b methyltransferase.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In one embodiment, the present invention relates to a zmet2a methyltransferase gene. The zmet2a methyltransferase gene of the present invention encodes a class II methyltransferase gene which controls CpNpG methylation. Nucleic acid sequences from the zmet2a methyltransferase gene described herein can be used to reduce or to alter the level of DNA methylation in a plant. In addition, the zmet2a nucleic acid sequence described herein can be used to methylate a targeted gene in a plant *in vivo* to "silence" or "knock-out" said gene.

In another embodiment, the present invention relates a zmet2b methyltransferase gene. The zmet2b methyltransferase gene can be isolated using a partial zmet2b methyltransferase gene described herein. Like the zmet2a methyltransferase gene, the zmet2b methyltransferase gene encodes a class II methyltransferase gene which controls CpNpG methylation. Nucleic acid sequences encoding the zmet2b methyltransferase gene can be used in the same manner as the nucleic acid sequence encoding the zmet2a methyltransferase gene to reduce or to alter the level of DNA methylation in a plant. In addition, the zmet2b nucleic acid sequence can be used to methylate a targeted gene in a plant *in vivo* to "silence" or "knock-out" said gene.

The present invention is applicable to a broad range of types of monocotyledonous and dicotyledonous plants, including, but not limited to, *Zea mays*, *Oryza sativa*. *Secale cereale*, *Triticum aestivum*. *Daucus carota*, *Brassica oleracea*, *Cucumis melo*. *Cucumis sativus*, *Latuca sativa*. *Solanum tubersoum*, *Lycopersicon esculentum*. *Phaseolus vulgaris*. and *Brassica napus*.

The nucleic acids of the present invention can be used in marker-aided selection. Marker-aided selection does not require the complete sequence of the gene or precise knowledge of which sequence confers which specificity. Instead, partial sequences can be used as hybridization probes or as the basis for oligonucleotide primers to amplify by PCR or other methods to follow the segregation of chromosome segments containing the zmet2a and/or zmet2b methyltransferase gene(s) in plants. Because the zmet2a or zmet2b methyltransferase marker is the gene itself, there can be negligible recombination between the marker and the methylated phenotype. Thus, the nucleic acids of the present invention can be used to provide an optimal means to DNA fingerprint class II DNA methyltransferases in other cultivars and wild germplasm. This can be used to indicate if other germplasm accessions and cultivars carry the same zmet2a and/or zmet2b methyltransferase genes.

Preparation of the Nucleic acids of the Present Invention

Generally, the nomenclature and the laboratory procedures involved with recombinant DNA technology described below are those well known and commonly employed by those of ordinary skill in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally, enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

The isolation of zmet2a and/or zmet2b methyltransferase gene(s) can be accomplished via a number of techniques. For instance, oligonucleotide probes based on the sequences disclosed herein can be used to identify the desired gene in a cDNA

or genomic DNA library. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. To prepare a cDNA library, mRNA is isolated from the desired organ of a particular plant, such as shoots from *Zea mays*, and a cDNA library which contains the zmet2a or zmet2b methyltransferase gene transcript is prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissues in which the zmet2a or zmet2b methyltransferase gene or homologs are expressed.

The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned zmet2a and/or zmet2b methyltransferase gene or partial sequence from either thereof (such as the partial zmet2b methyltransferase nucleic acid sequence shown in FIG. 23). Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species.

Those of ordinary skill in the art will appreciate that various degrees of stringency of hybridization can be employed in the assay; and either the hybridization or the wash medium can be stringent. As the conditions for hybridization become more stringent, there is a greater degree of complementarity required between the probe and the target for duplex formation to occur. The degree of stringency can be controlled by temperature, ionic strength, pH and the presence of a partially denaturing solvent such as formamide. For example, the stringency of hybridization is conveniently varied by changing the polarity of the reactant solution through manipulation of the concentration of formamide within the range of 0% to 50%.

Alternatively, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (hereinafter "PCR") technology can be used to amplify the sequences of the zmet2a and/or zmet2b methyltransferase and related genes directly from genomic DNA, from cDNA, from genomic libraries or from cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid

sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes.

The degree of complementarity (sequence identity) required for detectable binding will vary in accordance with the stringency of the hybridization medium and/or wash medium. The degree of complementarity will optimally be 100 percent; however, it should be understood that minor sequence variations in the probes and primers may be compensated for by reducing the stringency of the hybridization and/or wash medium as described earlier.

Appropriate primers and probes for identifying zmet2a and/or zmet2b methyltransferase nucleic acid sequences from plant tissues are generated from a comparison of the sequences provided herein. For a general overview of PCR see *PCR Protocols: A Guide to Methods and Applications.* (Innis, M, Gelfand, D., Snisky, J. and White, T., eds), *Academic Press*, San Diego (1990), incorporated herein by reference.

Nucleic acids may also be synthesized by well-known techniques as described in the technical literature. See e.g., Curruthers *et al., Cold Spring Harbor Symp. Quant. Biol.* 47:411-418 (1982), and Adams *et al., J. Am. Chem. Soc.* 105:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Proteins of the Present Invention

The present invention further provides for isolated zmet2a and/or zmet2b methyltransferases encoded by the zmet2a and/or zmet2b methyltransferase nucleic acids disclosed herein. One of ordinary skill in the art will recognize that nucleic acids encoding a functional zmet2a or zmet2b methyltransferase need not have a sequence identical to the exemplified genes disclosed herein. For example, because of codon degeneracy, a large number of nucleic acid sequences can encode the same

polypeptide. In addition, the polypeptides encoded by the zmet2a and/or zmet2b methyltransferase genes, like other proteins, have different domains which perform different functions. Specifically, zmet2a methyltransferase has ten (10) domains. These ten domains are identified as follows: I, chromodomain β2, chromodomain β3, IV, VI, VIII, IX and X. The ten domains and their sequence ranges (as shown in SEQ ID NO:2) are listed below in Table 1:

TABLE 1

Domain	Amino Acid Sequence Range
I	244-271
Chromodomain β2	366-379
Chromodomain β3	380-388
IV	411-434
VI	456-476
VIII	496-520
IX	723-746
X	751-775

Domains I and X are involved in binding AdoMet, which is source of the methyl group to be transferred during DNA methylation. Domain IV contains a catalytic domain. Domain VI aids in the positioning of domain IV. Domain VIII aids in DNA binding by neutralizing the charge of the phosphodiester backbone. The region between domain VIII and domain IX defines the sequence specificity of the zmet2a methyltransferase enzyme. Thus, the zmet2a methyltransferase gene sequences need not be full length, so long as the desired functional domain of the protein is expressed.

The zmet2a methyltransferase protein is at least 912 amino acid residues in length (see FIG. 2A), preferably, 932 amino acid residues in length (see FIG. 2B). However, those of ordinary skill in the art will appreciate that amino acid deletions, substitutions, or additions to the zmet2a methyltransferase protein will typically yield a enzyme possessing methylating characteristics similar or identical to that of the full length sequence. Thus, full length zmet2a methyltransferase proteins modified by 1,

2, 3, 4, or 5 deletions, substitutions, or additions, generally provide an effective degree of methylation relative to the full-length protein.

A partial amino acid sequence of the zmet2b methyltransferase protein is provided for in FIG. 24 and is 256 amino acids in length.

Modified protein chains can also be readily designed utilizing various recombinant DNA techniques well known to those of ordinary skill in the art. For example, the chains can vary from the naturally occurring sequence at the primary structure level by amino acid substitutions, additions, deletions, and the like. Modification can also include swapping domains from the proteins of the present invention with related domains from other class II methyltransferases.

The present invention also provides antibodies which specifically react with the zmet2a and/or zmet2b methyltransferase(s) of the present invention under immunologically reactive conditions. An antibody immunologically reactive with a particular antigen can be generated *in vivo* or by recombinant methods such as by selection of libraries of recombinant antibodies in phage or similar vectors. The term "immunologically reactive conditions" as used herein, includes reference to conditions which allow an antibody, generated to a particular epitope of an antigen, to bind to that epitope to a detectably greater degree than the antibody binds to substantially all other epitopes, generally at least two times above background binding, preferably at least five times above background. Immunologically reactive conditions are dependent upon the format of the antibody binding reaction and typically are those utilized in immunoassay protocols.

The term "antibody" as used herein, includes reference to an immunoglobulin molecule obtained by *in vitro* or *vivo* generation of the humoral response, and includes both polyclonal and monoclonal antibodies. The term also includes genetically engineered forms such as chimeric antibodies (e.g., humanized murine antibodies), heteroconjugate antibodies (e.g., bispecific antibodies), and recombinant single chain Fv fragments (scFv). The term "antibody" also includes antigen binding forms of antibodies (e.g., Fab', $F(ab')_2$, Fab, Fv, and, inverted IgG. See, Pierce

Catalog and Handbook. 1994-1995) Pierce Chemical Co., Rockford. IL). An antibody immunologically reactive with a particular antigen can be generated *in vivo* or by recombinant methods such as selection of libraries of recombinant antibodies in phage or similar vectors (*See*, *e.g.* Huse *et al.*, (1989) *Science* 246:1275-1281; and Ward, *et al.*, (1989) *Nature* 341:544-546; *and Vaughan* et al., (1996) *Nature Biotechnology*, 14:309-314).

Many methods of making antibodies are known to persons of ordinary skill in the art. A number of immunogens are used to produce antibodies specifically reactive to the zmet2a and/or zmet2b methyltransferase(s) of the present invention under immunologically reactive conditions. An isolated recombinant, synthetic, or native zmet2a and/or zmet2b methyltransferase(s) of the present invention is the preferred immunogens (antigen) for the production of monoclonal or polyclonal antibodies.

The zmet2a and/or zmet2b methyltransferase(s) is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies can be generated for subsequent use in immunoassays to measure the presence and quantity of the zmet2a and/or zmet2b methyltransferases. Methods of producing monoclonal or polyclonal antibodies are known to those of skill in the art (*See*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY).

Frequently, the zmet2a and/or zmet2b methyltransferase(s) and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

The antibodies of the present invention can be used to screen plants for the expression of the zmet2a and/or zmet2b methyltransferase(s). The antibodies of the present invention are also used for affinity chromatography in isolating zmet2a and/zmet2b methyltransferase(s).

The present invention further provides zmet2a and/or zmet2b methyltransferase polypeptides that specifically bind, under immunologically reactive conditions, to an antibody generated against a defined immunogen, such as an immunogen consisting of the polypeptides of the present invention. For example, immunogens will generally be at least 912 contiguous amino acids from the zmet2a methyltransferase polypeptide of the present invention. Nucleic acids which encode such cross-reactive zmet2a and/or zmet2b methyltransferase polypeptides are also provided by the present invention. The zmet2a/zmet2b methyltransferase polypeptides can be isolated from any number of plants as discussed earlier. Preferred plants are *Zea mays*, *Oryza* sativa, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

As used herein, the term, "specifically binds" includes reference to the preferential association of a ligand, in whole or part, with a particular target molecule (i.e., "binding partner" or "binding moiety" relative to compositions lacking that target molecule). It is, of course, recognized that a certain degree of non-specific interaction may occur between a ligand and a non-target molecule. Nevertheless, specific binding, may be distinguished as mediated through specific recognition of the target molecule. Typically, specific binding results in a much stronger association between the ligand and the target molecule than between the ligand and non-target molecule. Specific binding by an antibody to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. The affinity constant of the antibody binding site for its cognate monovalent antigen is at least 10^7, usually at least 10^9, more preferably at least 10^{10}, and most preferably at least 10^{11} liters/mole. A variety of immunoassay formats are appropriate for selecting antibodies specifically reactive with a particular protein. For example, solid-phase

ELISA immunoassays are routinely used to select monoclonal antibodies specifically reactive with a protein (See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific reactivity). The antibody may be polyclonal but preferably is monoclonal. Generally, antibodies cross-reactive to zmet2a and/or zmet2b methyltransferases are removed by immunoabsorbtion.

Immunoassays in the competitive binding format are typically used for cross-reactivity determinations. For example, an immunogenic zmet2a and/or zmet2b methyltransferase polypeptide is immobilized to a solid support. Polypeptides added to the assay compete with the binding of the antisera to the immobilized antigen. The ability of the above polypeptides to compete with the binding of the antisera to the immobilized zmet2a and/zmet2b methyltransferase polypeptides are compared to the immunogenic zmet2a and/or zmet2b methyltransferase polypeptide(s). The percent cross-reactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% cross-reactivity with such proteins as zmet2a and/or zmet2b methyltransferase(s) are selected and pooled. The cross-reacting antibodies are then removed from the pooled antisera by immunoabsorbtion with the non-zmet2a and/or non-zmet2b methyltransferase polypeptide(s).

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay to compare a second "target" polypeptide to the immunogenic polypeptide. In order to make this comparison, the two polypeptides are each assayed at a wide range of concentrations and the amount of each polypeptide required to inhibit 50% of the binding of the antisera to the immobilized protein is determined using standard techniques. If the amount of the target polypeptide required is less than twice the amount of the immunogenic polypeptide that is required, then the target polypeptide is said to specifically bind to an antibody generated to the immunogenic protein. As a final determination of specificity, the pooled antisera is fully immunoabsorbed with the immunogenic polypeptide until no binding to the polypeptide used in the immunoabsorbtion is detectable. The fully immunoabsorbed antisera is then tested for reactivity with the test polypeptide. If no reactivity is

observed, then the test polypeptide is specifically bound by the antisera elicited by the immunogenic protein.

Production of Recombinant Expression Cassettes

Isolated sequences prepared as described herein can then be used to provide recombinant expression cassettes. One of ordinary skill in the art will recognize that the nucleic acids used in the recombinant expression cassettes described herein encoding a functional zmet2a and/or zmet2b methyltransferase(s) need not have a sequence identical to the exemplified genes disclosed herein. In addition, the polypeptides encoded by the zmet2a and/or zmet2b methyltransferase genes, like other proteins, have different domains which perform different functions. Thus, the zmet2a and/or zmet2b methyltransferase gene sequences need not be full length, so long as the desired functional domain of the protein is expressed.

A DNA sequence coding for the desired zmet2a and/or zmet2b methyltransferase polypeptide(s), for example a cDNA or a genomic sequence encoding a full length protein, can be used to construct a recombinant expression cassette which can be introduced into a desired plant. An expression cassette will typically comprise the zmet2a and/or zmet2b methyltransferase nucleic acid(s) operably linked in either the sense or antisense direction to transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the zmet2a and/or zmet2b methyltransferase gene(s) in the intended tissues for the transformed plant.

For example, a plant promoter fragment may be employed which will direct expression of the zmet2a and/or zmet2b methyltransferase in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters includes the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1' or 2' - promoter derived from T-DNA of *Agrobacterium tumefaciens*, and ubiquitin other transcription initiation regions from various plant genes known to those of ordinary skill in the art.

Alternatively, the plant promoter may direct expression of the zmet2a and/or zmet2b methyltransferase gene in a specific tissue or may be otherwise under more precise environmental or developmental control. Such promoters are referred to here as "inducible" promoters. Examples of environmental conditions that may effect transcription by inducible promoters include pathogen attack, anaerobic conditions, or the presence of light.

Examples of promoters under developmental control include promoters that initiate transcription only in certain tissues, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may be fully or partially constitutive in certain locations.

The endogenous promoters from the zmet2a and/or zmet2b methyltransferase genes of the present invention can be used to direct expression of the genes. These promoters can also be used to direct expression of heterologous structural genes. The promoters can be used, for example, in recombinant expression cassettes to drive expression of genes to produce DNA methyltransferase in a particular cell or tissue.

To identify the promoters, the 5' portions of the clones described herein are analyzed for sequences characteristic of promoter sequences. For instance, promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs upstream of the transcription start site. In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element with a series of adenines surrounding the trinucleotide G (or T) N G. J. Messing et al., in *Genetic Engineering in Plants*, pp. 221-227 (Kosage, Meredith and Hollaender, eds. 1983).

If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the zmet2a or zmet2b methyltransferase coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences from the zmet2a and/or zmet2b methyltransferase gene(s) will typically comprise a marker gene which confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin. G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulforon.

As discussed above, the zmet2a and/or zmet2b methyltransferase gene(s) can be inserted into a recombinant expression cassette in the antisense direction. Expression of the zmet2a and/or zmet2b methyltransferase gene(s) in antisense direction will result in the production of antisense RNA. As is well known, a cell manufactures protein by transcribing the DNA of the gene encoding a protein to produce RNA. which is then processed to messenger RNA (mRNA) (e.g., by the removal of introns) and finally translated by ribosomes into protein. This process may be inhibited in the cell by the presence of antisense RNA. The term antisense RNA means an RNA sequence which is complementary to a sequence of bases in the mRNA in question in the sense that each base (or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. It is believed that this inhibition takes place by formation of a complex between the two complementary strands of RNA, thus preventing the formation of protein. How this works is uncertain: the complex may interfere with further translation, or degrade the mRNA, or have more than one of these effects. This antisense RNA may be produced in the cell by transformation of the cell with an appropriate DNA construct designed to transcribe the non-template strand (as opposed to the template strand) of the relevant gene (or of a DNA sequence showing substantial homology therewith).

The use of antisense RNA to downregulate the expression of specific plant genes is well known. Reduction of gene expression has led to a change in the phenotype of a plant, either at the level of gross visible phenotypic difference (e.g., lack of anthocyanin production in flower petals of petunia leading to colorless instead of colored petals (see van der Krol et al., *Nature*, 333:866-869 (1988)), or at a more subtle biochemical level, for example, a change in the amount of polygalacturonase

and reduction in depolymerization of pectin during tomato fruit ripening (Smith et al., *Nature*. 334:724-726 (1988)). Another more recently described method of inhibiting gene expression in transgenic plants is the use of sense RNA transcribed from an exogenous template to downregulate the expression of specific plant genes (Jorgensen, Keystone Symposium "Improved Crop and Plant Products through Biotechnology", Abstract X1-022 (1994)). Thus, both antisense and sense RNA have been proven to be useful in achieving downregulation of gene expression in plants, which are encompassed by the present invention.

<u>Production of Transgenic Plants</u>

Techniques for transforming a wide variety of higher plant species using the recombinant expression cassettes hereinbefore described are well known and described in the technical and scientific literature. See. for example. Weising *et al.*, *Ann. Rev. Genet.* 22:421-477 (1988).

The hereinbefore described recombinant expression cassettes may be introduced into the genome of a desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation, PEG poration, particle bombardment and microinjection of plant cell protoplasts or embryogenic callus, or the DNA constructs can be introduced directly to plant tissue using ballistic methods. such as DNA particle bombardment. In the alternative, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* host vector. The virulence functions of the *Agrobacterium* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria.

Transformation techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al.*, *EMBO J.* 3:2712-2722 (1984). Electroporation techniques are described in Fromm *et al.*, *Proc. Natl.*

Acad. Sci. USA 82:5824 (1985). Biolistic transformation techniques are described in Klein *et al.*, *Nature* 327:70-73 (1987).

Agrobacterium tumefaciens-mediated transformation techniques are well described in the scientific literature. See, for example Horsch *et al.*, *Science* 233:496-498 (1984), and Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* 80:4803 (1983). Although *Agrobacterium* is useful primarily in dicots, certain monocots can be transformed by *Agrobacterium*. For instance, *Agrobacterium* transformation of rice is described by Hiei *et al.*, *Plant J.*, 6:271-282 (1994).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the zmet2a and/or zmet2b methyltransferase nucleotide sequence(s). Plant regeneration from cultured protoplasts is described in Evans *et al.*, *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, pp. 124-176, MacMillian Publishing Company, New York, 1983; and Binding; *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee *et al.*, *Ann. Ref. of Plant Phys.* 38:467-486 (1987).

The methods of the present invention are particularly useful for incorporating the zmet2a and/or zmet2b methyltransferase nucleic acid(s) into transformed plants in ways and under circumstances which are not found naturally. In particular, the zmet2a and/or zmet2b methyltransferase(s) may be expressed at times or in quantities which are not characteristic of natural plants.

One of ordinary skill in the art will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

The hereinbefore described expression cassettes can be inserted into a plant in order to reduce or alter the amount of DNA methylation in a plant. Preferably, such an expression cassette contains the zmet2a and/or zmet2b methyltransferase gene(s) inserted into the cassette in the antisense direction as described earlier. A reduction or alteration in the amount of DNA methylation in a plant can be used to stabilize transgene expression in a transgenic plant.

One of the difficulties with the production of transgenic plants is that many transgenes are silenced or are not stable through successive generations. In many cases, transgene silencing is associated with increased DNA methylation. The hereinbefore described expression cassettes of the present invention containing the zmet2a and/or zmet2b methyltransferase gene(s) in the antisense direction can be inserted into a plant either before, concurrently with or after the insertion of another expression cassette containing a transgene which is to be expressed in the plant, such as, but not limited to, a resistance or drought tolerance gene, etc. The antisense RNA produced by the hereinbefore described expression cassette can then form a complex with the endogenous mRNA from the zmet2a and/zmet2b methyltransferase gene(s) within the plant. This complex should reduce or alter the amount of DNA methylation occurring *in vivo* in the plant. This reduction in DNA methylation should prevent the silencing of the desired transgene in the plant.

In a similiar manner, the expression cassettes described herein can be used to modify or alter the yield or biochemical qualities of a plant. As discussed earlier, certain genes in plants and animals are expressed differentially when transmitted thorough a male versus female parent. This phenomenon is known as imprinting. Imprinting is an epigenetic system correlated with DNA methylation. A reduction or alteration of DNA methylation in a plant by transforming a plant with an expression cassette containing the zmet2a and/or zmet2b methyltransferase gene(s) in the antisense direction may affect the yield and biochemical qualities of a plant.

The hereinbefore described expression cassettes can also be used to silence the expression of a particular targeted gene in plants *in vivo*. More specifically, the

expression cassettes of the present invention containing a tissue-specific promoter and the zmet2a and/or zmet2b methyltransferase gene(s) in the sense direction can be inserted into a plant. The tissue-specific promoter will direct expression of the zmet2a and/or zmet2b methyltransferase gene(s) in a area containing the desired targeted gene. Translation of the zmet2a and/or zmet2b methyltransferase gene(s) in the specific area will result in an increase in methylation in the area of the targeted gene. This increase in methylation can silence the targeted gene.

Transgenic plants containing the expression cassettes described herein and which exhibit a reduction in DNA methylation can be identified by using methylation sensitive restriction enzymes or High Performance Liquid Chromatography. Techniques for using methylation sensitive restriction enzymes and High Performance Liquid Chromatography are well known in the art. Transgenic plants containing the expression cassettes described herein and which exhibit an increase in DNA methylation can be identified by using a Northern Blot analysis which is well known in the art.

Additionally, the hereinbefore described expression cassettes can be used in gene therapy for human diseases which are caused by the amplification of trinucleotide repeats.

The following Examples are offered by way of illustration. not limitation.

EXAMPLES

EXAMPLE 1 -Cloning and Sequencing of Zmet2a

a. Cloning and Sequencing

A partial cDNA clone (CGET064) from an immature tassel cDNA library was obtained from Pioneer Hi-Bred International (Des Moines, Iowa). This clone was identified in an expressed tag sequence (hereinafter "EST") database using known DNA methyltransferase sequences for comparison. This original cDNA clone contained sequences from bp 151 to bp 2569 shown in FIG. 1A and 1B. The sequence of this clone, which represents the 3' end of the transcript was used to design forward and reverse primers for 5' and 3' Rapid Amplification of cDNA Ends

(hereinafter "RACE"). RACE was conducted using the Marathon cDNA Amplification Kit (available from Clontech) on cDNA prepared from Mo17 10 day old seedling mRNA. Mo17 is publically available from the National Seed Storage Lab (Fort Collins, Colorado). RACE products were isolated and ends sequenced using Marathon primers and gene specific primers. The remaining sequence was obtained from PCR products by primer walking. The primers used were AP2, 1F, 1R, 2R, 3R, 4F, 5F, 8R, 8F, 9R, 9F, 14F, 17F, and RaceRT (see FIG. 3). Two sequencing passes were made on the Mo17 cDNA ends and four sequencing passes were made on the intervening regions, three from Mo17 cDNA and one from B73. B73 is publically available from the National Seed Storage Lab (Fort Collins, Colorado). A consensus sequence for the coding region was generated and is shown in FIG. 1A and 1B.

Genomic sequence spanning primers 1F and 1R were obtained from Pioneer Hi-Bred International. To obtain the remaining genomic sequence of zmet2a, the CGET064 clone was used to probe a Mo17 genomic library (Stratagene). Lambda clones 4a, 4c, 4d1 and 4d2 were determined to be positive clones containing a sequence identical to CGET064. Lamda clone 4a did not contain the full length gene, therefore, sequence data was obtained from clone 4c. No analysis of clones 4d1 or 4d2 was conducted. Clone 4c was subcloned into pGEM7zf(+) (Promega) using double digests involving *Hind*III, *Xho*I, *Eco*RI, and *Bam*HI. Genomic sequence was obtained from a combination of subclones pHX8 (bp 7311-8878), pHX9 (bp 9173-10135), and pB11(bp 5269-8447) and by primer walking using primers T7, Sp6, M13F, M13R, Seq2FN, Seq2RN, S3F, S3R, 7F, 8eR, 9F, 9R, 11iR, 11iF, 12iR, 12iF, 13iR, 13iF, 14F, 14R, 15R, 15F, 16R, 16F, 17R, 17F, 18R, 18F, and RaceRT (see FIG. 3). Borders of the *Mu* insertion of zmet2a::MU1 were sequenced from PCR products using primer 5F and a *Mu* primer (see FIG. 3). Map locations of the zmet2a primers are shown in FIG. 5.

PCR products were sequenced using Big Dye terminator cycle sequencing on an ABI sequencer (Perkin-Elmer Applied Biosystems) at the University of Wisconsin Biotechnology Center Sequencing Facility (Madison, WI). Sequence data was processed using computional tools available through the World Wide Web (hereinafter, "WWW"), summarized in FIG. 6.

b. **Mutant Analysis**

A mutant allele called (zmet2a::Mu1) was obtained from Pioneer Hi-Bred International's TUSC system. This mutant allele contains a *Mutator* transposable element insertion and was identified in a *Mutator* population using a *Mu* specific primer and a zmet2a gene specific primer. Since the *Mutator* population is quite variable, heterozygous zmet2a::mu1 F_2 seed was advanced by selfing at the University of Wisconsin West Madison Agronomy Farm (Madison, Wisconsin), the University of Wisconsin Walnut Street greenhouses (Madison, Wisconsin), and at the University of Wisconsin winter nursery in Puerto Rico to produce the F_4 derived F_5 segregating family primarily used in this example.

DNA from 15 plants of the F_4 derived F_5 segregating family was used for HPLC analysis. A subset of these plants was used for Southern analysis. The 5^{th} to 7^{th} immature leaf tips were collected and immediately frozen in dry ice. Tissue was ground in liquid nitrogen and DNA was extracted using a modified CTAB method of Saghai-Maroof et al. (*Proc. Natl. Acad. Sci. USA* 81:8014-8018 (1984)). Tissue was incubated in CTAB (Sigma) extraction buffer for 2 hours at 65 °C, extracted with chloroform/isoamyl alcohol, treated with 0.5 mg RNase A (Sigma) for 30 minutes at 37 °C, extracted again with choroform/isoamyl alcohol, precipitated with isopropanol, washed with 10mM ammonium acetate/76% ethanol, and resuspended in TE.

Plants were genotyped by Southern analysis. DNA (10μg) was digested with *Bam*HI and *Eco*RI which cut on each side of the *Mu* insertion. The digested DNA was electrophoresed through a 0.8% agarose 0.5X TBE gel. DNA was transferred to Immobillon nylon membrane (Millipore) with 5X SSC. Blots were UV cross-linked for 25 seconds and dried at 80 °C for 1.5 hours. Pre-hybridization was carried out in 5X SSC, 50mM Tris pH 8.0, 0.2% SDS, 10 mM EDTA, 2.5X Denhardts solution, and 0.1 mg/ml single stranded sheared herring DNA overnight (8-16 hours) at 65 °C. Hybridization conditions were similar to pre-hybridization except for the addition of 5% dextran sulfate to the hybridization solution. Probes (25-50 ng) (clone CGET064 for genotyping) were radioactively labeled using a random priming reaction

containing 50 µCi of P-32 labeled dCTP. Following overnight hybridization at 65 °C, blots were washed 2X (0.15X SSC, 0.1% SDS) for 30-45 minutes at 65 °C. Hybridized blots were then exposed to Kodak Biomax film.

Southern analysis with methylation sensitive restriction enzymes was conducted in a similar manner except that 5 µg of DNA was digested. Enzymes included in the study were; *Apa*I, *Ava*II, *Bam*HI, *Bgl*II, *Bst*NI, *Cla*I, *Eco*O109, *Eco*RI, *Eco*RII, *Hae*III, *Hinf*I, *Hha*I, *Hpa*II, *Msp*I, *Pst*I, *Pvu*II, *Sac*I, *Sau*3a, *Scr*F1, *Sma*I, *Xho*I. Probes for repetitive sequence regions of the maize genome including a 9 kb clone for the maize 26s-5.8s-17s repeat (reviewed in McMullen et al., *Molecular Analysis of the Nucleolus Organizer Region in Maize. In: Chromosome Engineering in Plants: Genetics, Breeding, and Evoluation*. Gupta PK, Tsuchiya T. (eds). pp. 561-576 (1991)), the 5s ribosomal subunit clone (Mascia et al., *Gene*, 15:7-20 (1981)), and centromere probe pSau3a9 (Jiang et al., *Proc. Natl. Acad. Sci. USA* 93:14210-14213 (1996)) were used to analyze changes in methylation due to zmet2a::Mu1.

HPLC was conducted according to a modified protocol of Gehrke et al., (*J. Chromat*. 301:199-219 (1984)). Duplicate preparations for each of fifteen plants were analyzed. Twenty-five micrograms of DNA was diluted with water to a volume of 50 µl, denatured at 96 °C for 5 minutes and immediately placed on ice. One hundred microliters of 30mM ammonium acetate (pH 5.3), 5 µl of 20mM Zinc Sulfate and 10 µl Nuclease P1 (1mg/ml in 30mM ammonium acetate (pH 5.3) was added and incubated at 37 °C for 2 hours. This reaction cleaves 5' mononucleotides from single stranded DNA. The pH was adjusted with 20 µl of Tris (pH 8.5) and approximately 15 units of Calf Intestinal Alkaline Phosphatase was added and incubated at 37 °C for an additional 2 hours which converts the nucleotides to nucleosides. Samples were frozen at -20°C until HPLC analysis.

HPLC analysis was conducted at the University of Wisconsin Biotechnology Center. A volume of 50 µl was injected into a Brownlee Lab Spheri-5 RP-8 column. Nucleosides were separated with a flow rate of 0.75 ml/min using a gradient program consisting of 30 minutes in buffer A (0.05M Potassium Phosphate pH 4.0, 2.5% methanol), 19 minutes in buffer B (0.05M Potassium Phosphate pH 4.0, 20%

methanol). The column was flushed with 70% methanol for 13 minutes and then re-equilibrated with buffer A for 23 minutes before the injection of the next sample. All samples were analyzed on a Beckman System Gold chromatograph and nucleosides detected at A260nm and A280nm. Nucleoside and nucleotide standards (Sigma) were used to determine nucleoside peak positions and to create a standard curve to determine nucleoside concentration. The ratio of 5-methylcytosine to total cytosine was calculated and statistical analysis conducted using SAS.

To test remethylation as an indication of *de novo* methylase activity, an F_1 hybrid of an F_4 line homozygous for zmet2a::Mu1 and the inbred line Mo17 was backcrossed to the nonmutant Mo17 parent to generate plants homozygous wild-type and plants heterozygous for zmet2a::Mu1. Seedlings of the F_1, the BC_1 progeny, the Mo17 parent and a sib of the F_4 zmet2a::Mu1 parent were grown in the greenhouse and DNA extraction and Southern analysis conducted as previously described. DNA was digested with *Msp*I and *Pst*I and probed with the aforementioned repetitive clones.

c. Expression Analysis

The expression of zmet2a was determined by hybridizing the zmet2a cDNA probe to a Southern blot of cDNA's prepared from different tissues and tissues at different stages of development. Tissues included in this study are embryos 24 days after pollination, 10 day seedlings, immature ear, immature tassel, immature leaf from mutant and nonmutant plants, and roots. Total RNA was extracted using Trizol (Gibco/BRL) according to the manufacture's protocol. The PolyAttract System (Promega) was used to isolate mRNA's from all tissues except 10 day seedlings which was isolated using oligo dT cellulose columns (Pharmacia). cDNA was synthesized from the isolated RNA's using Marathon cDNA Amplification Kit (Clontech).

d. Results

zmet2a shares sequence similarity with other DNA methyltransferases

zmet2a is a member of a small gene family. Three cohybridizing bands are observed on a Southern blot of B73 DNA digested with *Hind*III and probed with

clone CGET064 which does not contain a *Hind*III restriction site (see FIG. 7). zmet2a, which maps to the long arm of chromosome 10, is coded on 20 exons with 19 intervening introns (FIG. 5). The inferred protein using the first predicted translation start site located within a consensus Kozak sequence (Kozak, *J. Cell. Biol.*, 115:887-903 (1991)) is composed of at least 912 amino acids with a predicted mass of 101 Kd (Kilodaltons). A protein of this size with an affinity for CpNpG sequences was isolated in *Pisum sativum* by Pradhan and Adams (*Plant J.*, 471-481 (1995)).

Comparisons with *Arabidopsis* chromomethylase, *CMT1*

Sequence of zmet2a (FIG. 1A and 1B) reveals that it lacks the large N-terminal domain found in the maintenance enzymes but does possess the six highly conserved motifs of the C-terminal catalytic domain. Database searches using BLAST (http://www.ncbi.nlm.nih.gov/gov/BLAST/) show that zmet2a has highest sequence homology to the *Arabidopsis* chromomethylase, *CMT1* (see Henikoff and Comai, *Genetics*, 148:307-318 (1998)) with 44% identity, 57% conservation. The N-terminal region is larger in zmet2a; however, there is an additional downstream predicted start site, also within a consensus Kozak sequence, that codes for an enzyme of 809 amino acids which is more similar in size to the most closely related *CMT1* which is composed of 791 amino acids.

Alignments of zmet2a with *CMT1* and the catalytic domains of *Arabidopsis MET1* and maize *zmet1* maintenance enzymes show conservation in the important functional motifs I, IV, VI, VIII, IX and X providing evidence that it is indeed a DNA methyltransferase (FIG. 8). zmet2a and *CMT1* are 87% conserved across the defined six conserved domains, as shown in the underlining in FIG. 8. Zmet2a and CMT1 also have 60% conservation in the variable region sequence between the defined underlined motifs VIII and IX in FIG. 8, which contains a region known as the target recognition domain in the bacterial methyltransferases. The bacterial methylase M.*Hha*I has been crystalized and functions deduced for the conserved amino acids (Cheng et al., *Cell*, 74:299-307 (1993)). The zmet2a amino acids involved in catalysis were predicted by comparison to M.HhaI. The amino acids interacting with SAM and with cytosine are summarized in FIG. 9.

zmet2a mutant plants have reduced methylation at CpNpG sites

A reverse genetics approach was used to ascertain the function of zmet2a. A F_2 family segregating for a *Mutator (Mu)* insertion in the exon encoding motif IX was identified using a PCR primer for *Mu* and a gene-specific primer for zmet2a. This allele is called zmet2a::Mu1. The insertion of *Mu* into exon 19 results in a transcript that would code for a protein truncated at the point of the *Mu* insertion in motif IX due to the introduction of a stop codon. The resulting protein is expected to be dysfunctional since it lacks Motif X which is required for S-Adenosyl methionine (hereinafter "SAM") binding (Cheng et al. *Cell*, 74:299-307 (1993)).

Reduced methylation observed by restriction enzyme analysis

To reduce the genetic background variation associated with the heterogeneous origin of the *Mutator* population. restriction enzyme analysis was conducted on a F_4 derived F_5 family segregating for zmet2a::Mu1. Restriction enzyme isoschizomers *Hpa*II/*Msp*I in addition to other methylation sensitive enzymes were used to determine methylation pattern differences among the three genotypic classes. *Hpa*II and *Msp*I both recognize the sequence CCGG but differ in their sensitivity to methylation. *Hpa*II digestion is inhibited unless both cytosines are unmethylated whereas *Msp*I can digest $C^{me}CGG$ sequences but not $^{me}CCGG$ sites. The methylation status at CpG sites can be accessed by digesting with *Hpa*II and similarly *Msp*I digestion is used to determine the state of methylation at CpCpG sites specifically and may provide a general indication of methylation changes occurring at CpNpG sites.

Results indicate significant reductions in cytosine methylation at ^{me}CCG sites as indicated by a more complete digestion by *Msp*I in plants homozygous for zmet2a::Mu1 (FIG. 10 A-C). Plants heterozygous for zmet2a::Mu1 were intermediate in their digestion pattern. Although the frequency of methylated cytosines is much higher at CpG sequences. no changes in methylation were observed among the genotypic classes when digested with *Hpa*II (FIG. 10 A-C).

Isoschizomers, *Bst*NI and *Eco*RII recognize the sequence CC(A/T)GG. *Bst*NI is not sensitive to cytosine methylation and *Eco*RII is inhibited at $C^{me}C$(A/T)GG sites. Nearly all of these sites are methylated in repetitive sequences as a low level of

*Eco*RII digestion is observed only in zmet2a::Mu1 plants (See FIG. 11), whereas digests with *Bst*NI are completely digested to lower molecular weight fragments for all genotypes. These methylated sites may not be subject to zmet2a activity but may instead be methylated by another member of the zmet2a gene family or by *zmet1* or possibly *de novo* methylated after each cell cycle by *zmet3*. Other restriction enzymes were used to clarify the apparent sequence specificity of methylation reduction at CpNpG sites. As with the isoschizomers, no digestion differences are observed with CpG sensitive enzymes *Hha*I [G^{me}CGC] and *Cla*I [AT^{me}CGAT]. More complete digestion is observed in plants homozygous for zmet2a::Mu1 with enzymes sensitive to methylation at CpNpG sites. FIG. 12 shows digestion patterns for enzymes sensitive to methylation at CpNpG sites: *EcoR*II, *Bgl*II, *Pst*I, *Bam*HI, and *Ava*II. In addition to *Eco*RII as previously mentioned, reduced methylation in one or more of the repetitive regions was observed with *Bgl*II [AGAT^{me}CT], *Pst*I [^{me}CTGCAG], *Bam*HI [GGAT^{me}CC], and *Ava*II [GG(A,T)^{me}C^{me}C]. It should be noted that *Ava*II may include some CpG overlapping sites. Subtle differences in digestion patterns of one or more of the repetitive sequences were also observed with *Sau3a*I [GAT^{me}C], *Apa*I [GGG^{me}CC^{me}C], and XhoI [^{me}CT^{me}CGAG]. With these enzymes it is not possible to unambiguously determine whether the source of the difference is CpG or CpNpG methylation. Differences were also observed with *Scr*FI [C^{me}CNGG] which duplicates the targeted sequences and methylation sensitivities of *Eco*RII, *Msp*I and *Hpa*II. Although in many cases the observed reduction in CpNpG or CpN methylation is minimal, any cases of reduced methylation that could be unambiguously attributed to CpG sites have not been observed.

Reduced methylation observed by HPLC

To further assess the extent of methylation reduction caused by the zmet2a::Mu1 allele, HPLC was used to determine the proportion of methylated cytosines in the same F_5 plants used for restriction enzyme analysis. An 11.6% decrease in 5-methylcytosine was observed in plants homozygous for zmet2a::Mu1 relative to siblings homozygous for wild-type zmet2a (FIG. 12). Heterozygotes were intermediate in 5-methylcytosine content. Differences between the genotypic classes are statistically significant at $\alpha < 0.0001$. Since most methylation is found at CpG sites (Gruenbaum et al., *Nature*, 292:860-862 (1981)), a 12% decrease in the total 5-

methylcytosine content likely accounts for a substantial reduction in methylation at CpNpG sites if the reductions are confined to these sequences.

Several generations of inbreeding does not reduce methylation levels beyond that which is observed in the F_2 homozygous mutant (FIG. 13). In addition, it was also observed that plants restored to a normal zmet2a genotype from zmet2a::Mu1 heterozygotes appeared to have near normal levels of methylation.

Methylation is restored after segregation away from zmet2a::Mu1

To test remethylation, a nonmutant line, Mo17, was crossed to a homozygous mutant line, the resulting F_1 was then backcrossed to the nonmutant Mo17 parent line. Restriction enzyme analysis of backcross progeny show all individuals without the *Mu* insertion have remethylated to levels similar to the backcross parent (see FIG. 14). The increased levels of methylation observed in normal BC_1 progeny appear to be higher than that expected from the segregation of normal Mo17 derived chromosome segments and low methylation mutant segments, which would result in a pattern intermediate between the F_1 and the nonmutant parent. These results indicate either that zmet2a has *in vivo de novo* activity and is responsible for establishing CpNpG methylation patterns, or that a separate *de novo* methyltransferase functions only early in development and that zmet2a is responsible for maintaining these patterns. These results on remethylation are in contrast to those of the reduced methlyation patterns of *Arabidopsis* mutants. Backcross progeny, lacking an antisense *MET1* transgene or the *ddm1* mutation, derived from mutant plants outcrossed to normal plants showed very slow remethylation and required several generations to restore methylation to normal levels (Ronemus et al., *Science*, 273:654-657 (1996), Vongs et al., *Science*, 260:1926-1928 (1993), Kakutani et al., *Genetics*, 151:831-838 (1999)). Similar results were observed in selfed progeny from hemizygous antisense *MetI* plants that did not inherit the transgene (Finnegan et al., *Proc. Natl. Acad. Sci. USA* 93:8449-8454 (1996)) however a centromeric region and some single copy sites did remethylate in the first generation (Finnegan et al., *Annu. Rev. Plant Physiol. Plant Mol. Bio.*, 49:223-247 (1998)).

Other DNA methyltransferases that lack the large N-terminal domain have been presumed to be *de novo* enzymes, however, evidence remains insufficient. *In vitro* expression of *Dnmt*3a and *Dnmt*3b (Okano et al., *Nature Genetics*, 19:219-220 (1998)) did not show a specific preference for hemimethylated DNA or nonmethylated DNA and in vivo expression in *Drosophila* (Lyko et al., *Nature Genet.*, 23:363-366 (1999)) further confirm *de novo* activity, whereas *Dnmt*2 (Okano et al., *Nucleic Acids Res.*,26:2536-2540 (1998)) was shown not to effect *de novo* or maintenance methylation in mice. *Masc1*, in *ascobolus*, is purported to have *de novo* activity through its effect on methylation induced premeiotically (MIP) (Malagnac et al., *Cell*, 91:281-290 (1997)). Another Ascobolus methyltransferase *Masc2* was found to be dispensible for maintenance and de novo methylation *in vivo* (Malagnac et al, *Mol. Micro.* 3:331-338 (1999)).

A chromodomain is present in zmet2a

A distinguishing feature of zmet2a, like *CMT1*, is the presence of the chromodomain. Chromodomains have been demonstrated to target proteins to heterochromatic regions and may also be a site of protein-protein interactions (reviewed by Cavalli and Paro, *Curr. Op. Cell Biol.*, 10:354-360 (1998)). The presence of the chromodomain in zmet2a and *CMT1* potentially suggests targeting of the methyltransferase to chromatin complexes or a role of the methyltransferase in chromatin formation and stability. Furthermore, the observation that zmet2a affects CpXpG methylation may also implicate protein targeting through the chromodomain and targeting of methylation patterns. Stable transcriptionally active or silent states may be determined by the formation of chromatin complexes. The mechanisms involved in the formation of silencing complexes remain unknown. However, there is evidence of the involvement of methylation in transcriptionally silenced states which involve methylation binding proteins, transcriptional repressor complexes, and histone deacetylases (Nan et al., *Nature*, 393:386-389 (1998), Wade et al, *Nature Gen.*, 23:62-66 (1999), Ng et al., *Nature. Gen.* 23:58-61 (1999)).

zmet2a is expressed throughout plant development. Expression is higher in the rapidly dividing tissues of seedling, immature ear and embryos (FIG. 15) consistent with the role of methyltransferases in methylating newly synthesized DNA.

Low expression of zmet2a in terminal tissue (leaves) could serve a protective function against invading DNA if this enzyme does have a *de novo* function.

Example 2 – Cloning and Sequencing of the maize retrotransposon SPRITE-1

This example describes the cloning and sequencing of a maize retrotransposon that is inserted into an intron of zmet2a and is referred to herein as "SPRITE-1".

a. Introduction

Within the genomes of most organisms are DNA elements that can be considered parasitic. These elements confer no phenotype of their own and function only for their propagation and insertion elsewhere in the genome. There are two major classes of these elements based on the mechanisms of propagation. One class propagates using DNA-mediated mechanisms where the element does not code for any polymerase and entirely depends on the replication machinery of the host. This class includes the *Ac*, *Spm*, and *Mu* transposable element systems. The other major class is known as retrotransposons, retrotransposable elements or retroelements (reviewed in Grandbastien, *Trends in Genetics* 8:103-108 (1992); Eickbush, *Origin and Evolutionary Relationships of Retroelements. In The Evolutionary Biology of Viruses* (Morse, S.S., ed).) (1994); Wessler et al., *Current Biology*, 5:814-821 (1995); Bennetzen, *Genome*, 37:565-576 (1996)). These elements are not able to excise from one site and insert into another, as the previously mentioned class is capable, but replicate by an RNA-mediated process. The retroelements code for a reverse transcriptase which is a DNA polymerase that uses RNA as a template.

There are several types of retroelements. The main types are retroviruses, long-terminal-repeat (hereinafter "LTR") retroelements, and non-LTR retroelements. Retroviruses are infectious and have not been found in plants, although one plant LTR-retroelement, SIRE-1 from soybean has coding sequences similar to that of a retoviral envelope protein (Laten et al., *Proc. Natl. Acad. Sci.*, 95:6897-6902 (1998)). The non-LTR class is mainly composed of long interspersed nuclear elements (hereinafter "LINEs") and short interspersed nuclear elements (hereinafter "SINEs"). These elements have been found in plants. Less is known about this class than the others. They do differ from LTR-retroelements in that they contain a poly-A tail at

their 3' end. The LTR-retroelement class has been more extensively described in plants than the other classes of retroelements. The LTR-retroelements are usually categorized as one of two groups based on the similarity with the first elements described in yeast and *Drosophila*. One group shares similarity with the Ty3 elements from yeast and the *gypsy* element of *Drosophila* (Marlor et al., *Mol. Cell. Biol.*, 22:829-846 (1986); Clark et al.. *J. Biol. Chem.*, 263:1413-23 (1988)). The other group has similarity with the Ty1 elements of yeast and the *copia* element of *Drosophila*. The element identified in this study is of the Ty1/*copia* class (Clare and Farabaugh, *Proc. Natl. Acad. Sci. USA*, 82:2829-2833 (1985); Mount and Rubin, *Mol. Cell. Biol.* 5:1630-1638 (1985)).

The general structure of a LTR-retroelement is depicted in FIG. 16A. These elements are similar in their structure and replication to retroviruses (reviewed in Witcomb and Hughes, *Ann. Rev. Cell Biol.*, 8:275-306 (1992), Eickbush, *Origin and Evolutionary Relationships of Retroelements*. In *The Evolutionary Biology of Viruses* (Morse, S.S., ed.). New York: Raven Press, pp 121-157 (1994), Bennetzen, *Trends in Microbiology*, 9:347-353 (1996)). These elements have direct repeats at the termini as opposed to the DNA based elements that have inverted terminal repeats. Downstream from the 5' LTR is a primer binding site for a host tRNA that primes the first DNA strand synthesis using reverse transcriptase. One or more open reading frames that code for *gag*, a protease, an integrase, a reverse transcriptase, and RNaseH are located downstream from the primer binding site. After the coding region is a polypurine tract followed by the 3' LTR. Ty3/*gypsy* and Ty1/*copia* elements differ in the postion of the integrase coding region. Ty3/gypsy element have the integrase domain at the end of the coding region whereas Ty1/*copia* element have it positioned between the proteinase and reverse transcriptase regions. The *gag* gene encodes proteins for the nucleocapsid and the highly conserved cysteine-histidine nucleic acid binding domain ($CX_2CX_4HX_4C$). The protease processes the polyprotein into its individual components. The integrase functions to insert a newly replicated element into the host DNA. The reverse transcriptase synthesizes the first DNA strand from the transcribed RNA of the element. The RNase degrades the RNA following first strand synthesis. Retroelements rely on the RNA polymerase of the host for

transcription and the host DNA polymerase for second strand DNA synythesis to complete replication.

Using PCR based methods. retroelements were found within nearly every species of the plant kingdom studied (Flavell et al., *Nuc. Acids Res.* 20:3639-3644 (1992); Voytas et al., *Proc. Natl. Acad. Sci. USA* 89:7124-7128 (1992)). Despite the ubiquitous nature of retroelements. there is great heterogeneity among the element within and among species (Flavell et al., *Nuc. Acids Res.* 20:3639-3644 (1992). Wang et al., *Plant Mol. Biol.*, 33:1051-1058 (1997), Pearce et al., *Mol. Gen. Genet.*, 250:305-315 (1996)).

Retroelements are found to be distributed over the entire lengths of chromosomes in *Avena sativa* (Katsiotis et al., *Genome*, 39:410-417 (1996)) but have also been found to be less abundant in heterochromatin, nucleolar organizer regions, centromeres and telomeres (Pearce et al., *Mol. Gen. Genet.*, 250:305-315 (1996); Moore et al., *Genomics*, 10:469-476 (1991); Aledo et al., *Theor. Appl. Genet.*, 90:1094-1100 (1995); Brandeis et al., *Plant Mol. Biol.*, 33:11-21 (1997)). Retroelement-like sequence were found in centromeric regions of grass chromosomes (Miller et al., *Genetics*, 150:1615-1623 (1998)). Many retroelements were discovered by their associations with plant genes (Johns et al., *EMBO J.*, 4:1093-1102 (1985); Grandbastien et al., *Nature*, 337:376-380 (1989); Camirand et al., *Mol. Gen. Genet.*, 224:33-39 (1990)); White et al., *Proc. Natl. Acad. Sci. USA*, 91:11792-11796 (1994)); Hu et al., *Mol. Gen. Genet.*, 248:471-480 (1995); Bi and Laten, *Plant Mol. Biol.*, 30:1315-1319 (1996), Royo et al., *Mol. Gen. Genet.*, 250:180-188 (1996); Kumekawa et al., *Mol. Gen. Genet.*, 260:593-602 (1999)). Many more retroelements or retroelement fragments have been identified using PCR with degenerate primers (Voytas et al., *Proc. Natl. Acad. Sci. USA*, 89:7124-7128 (1992)); Flavell et al., *Nuc. Acids Res.*, 20:3639-3644 (1992); Flavell et al., *Mol. Gen. Genet.*, 231-233 (1992), Pearce et al., *Mol. Gen. Genet.*, 250:305-315 (1996); Katsiotis et al., *Genome*, 39:410-417 (1996); Wang et al., *Plant Mol. Biol.*, 33:1051-1058 (1997)). Others have been identified through studies for other purposes (Bhattacharyya et al., *Plant Mol. Biol.*, 34:255-264 (1997); Vicient and Martinez-Izquierdo, *Gene*, 184:257-261 (1997);

Manninen and Schulman, *Plant Mol. Biol.*, 22:829-846 (1993)) or by genome sequencing projects.

The Ty3/*gypsy* and the Ty1/*copia* elements can be found in large numbers and may contribute up to 50% of the nuclear DNA of the maize genome (SanMiguel et al., *Science*, 274:765-768 (1996)). A 280 Kb region of the maize genome containing the Adh1-F and u22 genes was composed of retroelements, from 10 different families, inserted within each other. The copy number of Ty1/*copia* elements varies considerably. For example, the Ta1 elements of *Arabidopsis* (Voytas et al., *Genetics*, 126:713-721 (1990)) and the Tst1 element of *Solanum tuberosum* (Camirand et al., *Mol. Gen. Genet.*, 224:33-39 (1990)) have one to only a few copies whereas the maize element PREM-2 (Bennetzen, *Trends in Microbiology*, 9:347-353 (1996)) and the BARE-1 element of *Hordeum vulgare* (Manninen and Schulman, *Plant Mol. Biol.*, 22:829-846 (1993)) may be present at 30,000 or more copies.

The differences in copy number infer differences in expression of retroelements. Retroelements are not expressed at high levels as only a few examples of activity have been observed. The Bs1 and Zeon-1 elements of maize (Johns et al., *EMBO J.*, 4:1093-1102 (1985); Hu et al., *Mol. Gen. Genet.*, 248:471-480 (1995)); the Tos elements of rice (Hirochika et al., *Proc. Natl. Acad. Sci. USA* 93:7783-7788 (1996)) the Tnt1 and Tto1 elements of tobacco (Grandbastien et al., *Nature*, 337:376-380 (1989); Hirochika, *EMBO J.*, 12:2521-2528 (1993)) and the Tnp2 element of *Nicotiana plumbaginifolia* have shown evidence of activity. Retroelement expression is higher in plant tissues under stressful conditions. The Tto1, Tto2 of tobacco and Tos17 element of rice were shown to be activated in tissue culture (Hirochika, *EMBO J.*, 12:2521-2528 1993, Hirochika et al., *Proc. Natl. Acad. Sci.*, USA (1996)). The promotors of the BARE-1 element of barley and the Tnt-1 element of tobacco drove expression of reporter genes in protoplasts (Suoniemi et al., *Plant Mol. Biol.*, 31:295-306 (1996); Pouteau et al., *EMBO J.*, 10:1911-1918 (1991)).

Biotic stresses such as viral, fungal and bacterial infection and abiotic stress such as wounding have also been shown to initiate the expression of Tnt1 and Tto1 retroelements (Pouteau et al., *Plant J.*, 5:535-542 (1994); Moreau-Mhiri et al., *Plant*

J., 9:409-419 (1996); Vernhettes et al., *Plant Mol. Biol.*, 35:673-679 (1997); Mhiri et al., *Plant Mol. Biol.*, 33:257-266 (1997); Grandbastien et al, *Genetica*, 100:241-252 (1997); Takeda et al., *Plant Mol. Biol.*, 36:365-376 (1998)). The Bs1 element of maize may have been mobilized prior to insertion in the Adh1 gene by infection with the barley stripe mosaic virus (Johns et al., *EMBO J.*, 1093-1102 (1985)). Only the expression of BARE-1 has been observed in normal unstressed barley leaves (Suoniemi et al., *Plant Mol.. Biol.*, 31:295-306 (1997)).

Under normal conditions, retroelements are transcriptionally inactive and are thus transpositionally inactive. Mechanisms within the host must exist to regulate the activity of the retroelements to prevent potentially deleterious mutations that could occur if retroelement transposition was unchecked. Most retroelements are highly methylated (Bennetzen et al., *Genome*, 37:565-576 (1994)) and possibly in heterochromatic regions and may not be accessible to transcriptional machinery. Though silenced in most cases and active in stressful situations, it has been suggested that retroelement transposition may create mutations that may be of selective advantage and provide a means for adaptation (McClintock, *Science*, 226:792-801 (1984)).

b. Cloning and Sequencing of SPRITE-1.

A zmet2a genomic clone was isolated from a lambda library (Stratagene) constructed from Mo17 genomic DNA. The sequence was obtained from subclones or from PCR products by primer walking. Fragments were sequenced using Big Dye terminator cycle sequencing on an ABI sequencer (Perkin-Elmer Applied Biosystems) at the University of Wisconsin Biotechnology Center Sequencing Facility, Madison, Wisconsin.

Expression analysis was conducted on cDNA's prepared using Marathon cDNA Amplification Kit (Clontech) according to the manufacturer's protocols from mRNA isolated from a Mo17 10 day old seedling, Mo17 immature tassel, B73 immature ear, Black Mexican Sweet (BMS) callus, Mo17 embryo 24 days after pollination, W22 pollen, young roots, and immature leaf tissue from zmet2a normal and mutant plants. Total RNA was extracted using Trizol (Gibco/BRL) according to

manufacturer's protocol. Seedling mRNA was isolated using oligo dT cellulose columns (Pharmacia) all other mRNA isolated using the PolyAttract system (Promega).

c. DNA extraction and Southern analysis for genotyping and methylation analysis.

DNA was extracted from immature leaf blades as described in Saghai Maroof et al. (*Proc. Natl. Acad. Sci. USA* 81:8014-8018 (1984)). The copy number of SPRITE-1 was determined by digesting DNA (10μg) with *Eco*RI which does not cut within the element. The digested DNA was electrophoresed through a 0.8% agarose 0.5X TBE gel. Gels were treated with 0.25N HCl for 15 minutes, denatured in 0.2N NaOH and 0.6 M NaCl for 30 minutes, then neutralized in 0.5 M Tris 1.5 M NaCl for 30 minutes. DNA was transferred to Immobilon nylon membrane (Millipore) with 5X SSC. Blots were dried at 80 °C for 1.5 hours. Pre-hybridization was carried out in 5X SSC, 50 mM Tris pH 8.0, 0.2% SDS, 10 mM EDTA, 2.5X Denhardt's solution, and 0.1 mg/ml single stranded sheared herring DNA overnight (8-16 hours) at 65 °C. Hybridization conditions were similar to pre-hybridization except for the addition of 5% dextran sulfate to the hybridization solution. The blot was probed with a PCR fragment (25-50 ng) amplified from the 5' end of the element. Probes were P-32 (50 μCi) labeled using random priming. Following overnight hybridization at 65 °C, blots were washed 2X (0.15X SSC, 0.1% SDS) for 30-45 minutes at 65 °C. Hybridized blots were then exposed to Kodak BioMax film. Southern analysis with methylation sensitive restriction enzymes was conducted on B73 and Mo17 using the same protocols as for genotyping except that 5 μg of DNA was digested. Enzymes included in the study were the differentially methylation sensitive isoschizomers *Hpa*II/*Msp*I and *Eco*RII/*Bst*NI as well as other methylation sensitive enzymes: *Hha*I, and *Pst*I. Blots were hybridized with probes representing different portions of the element.

d. HPLC analysis.

HPLC was conducted according to a modified protocol of Gehrke et al. (*J. Chromato.*, 301:199-219 (1984)). B73 x Mo17 recombinant inbred lines carrying a SPRITE-1 insertion were determined using PCR with the zmet2a primers 15F and 8R,

and the SPRITE-1 primer 18R. Preparations for each of four plants with and without SPRITE-1 were analyzed. Twenty-five micrograms of DNA was diluted with water to a volume of 50 µl, denatured at 96 °C for 5 minutes and immediately placed on ice. One hundred microliters of 30 mM ammonium acetate (pH 5.3), 5 µl of 20 mM Zinc Sulfate and 10 µl Nuclease P1 (1mg/ml in 30 mM ammonium acetate (pH 5.3) was added and incubated at 37 °C for 2 hours. This reaction cleaves 5' mononucleotides from single stranded DNA. The pH was adjusted with 20 µl of Tris (pH 8.5) and approximately 15 units of Calf Intestinal Alkaline Phosphatase was added and incubated at 37 C for an additional 2 hours which converts the nucleotides to nucleosides. Samples were frozen at -20 °C until HPLC analysis.

HPLC analysis was conducted at the University of Wisconsin Biotechnology Center, Madison, Wisconsin. A volume of 40 µl was injected into a Brownlee Lab Spheri-5 RP-8 column. Nucleosides were separated with a flow rate of 0.75 ml/min using a gradient program consisting of 30 minutes in buffer A (0.05M Potassium Phosphate pH 4.0, 2.5% methanol), 19 minutes in buffer B (0.05M Potassium Phosphate pH 4.0, 20% methanol). The column was flushed with 70% methanol for 13 minutes and then re-equilibrated with buffer A for 23 minutes before the injection of the next sample. All samples were analyzed on a Beckman System Gold chromatograph and nucleosides detected at A260 nm and A280 nm. Nucleoside and nucleotide standards (Sigma) were used to determine nucleoside peak positions and to create a standard curve to determine nucleoside concentration. The ratio of 5-methylcytosine to total cytosine was calculated and statistical analysis conducted using SAS.

e. **Expression analysis.**

The expression of SPRITE-1 was determined by hybridizing a SPRITE-1 probe to a Southern blot of cDNA's prepared from different tissues and tissues at different stages of development. Tissues included in this study are embryos 24 days after pollination, 10 day seedlings, immature ear, immature tassel, immature leaf from mutant and nonmutant plants, roots, BMS callus, and mature pollen. Total RNA was extracted using Trizol (Gibco/BRL) according to the manufacture's protocol. The PolyAttract System (Promega) was used to isolate mRNA's from all tissues except 10

day seedlings which was isolated using oligo dT cellulose columns (Pharmacia). cDNA was synthesized from the isolated RNA's using Marathon cDNA Amplification Kit (Clontech).

f. Results

SPRITE-1 is similar to retrotransposons of the Ty1/*copia* group.

In the process of sequencing the maize methyltransferase gene zmet2a, a retroelement inserted within an intron of this gene was discovered and names SPRITE-1. This element is positioned in opposite transcriptional orientation relative to zmet2a. The insertion spans 5220 bp and possesses all the components of a retroelement. Sequence data indicates that SPRITE-1 is a Long-Terminal-Repeat (hereinafter "LTR") retroelement belonging to the Ty1/*copia* class of retroelements. FIG. 16a depicts the general structural components of SPRITE-1. FIG. 16b shows the sequence of the terminal structural components. SPRITE-1 has a perfect 109 bp direct terminal repeats which includes a 3 bp inverted repeat that flanks the internal element sequence. These repeats have the TG...CA pattern found in most plant retroelements and are also shorter than LTR's of most retroelements. LTR's range in size from 115 bp to 4560 bp from information compiled by Bennetzen (*Trends in Microbiology*, 9:347-353 (1996)). A 5 bp host site duplication flanks the repeats externally. Downstream and adjoining the 5' LTR is a primer binding site (PBS) of 16 bp that has sequence complementary to the wheat germ cytoplasmic initiator methionine tRNA (Ghosh et al., *Nuc. Acids. Res.*, 10:3241-3247 (1982)). Upstream and adjoining the 3' LTR is a polypurine tract of 9 bp. Between the putative transcription start site to the predicted translation start site is a 550 bp untranslated region. SPRITE-1 contains a single open reading frame coding 1485 amino acids ending with the stop codon at the 5' end of the polypurine tract.

Database searches for similar coding sequences using BLAST (http://www.ncbi.nlm.nih.gov/gov/BLAST/) show that SPRITE-1 belongs to a different family of retroelements than any other previously described. The most closely related elements based on overall amino acid similarity include an *Arabidopsis* retroelement (AC006528), Retrofit from *Oryza longstaminata* (U72725), and Hopscotch from *Zea mays* (U12626) all having ~35% identity and ~50%

conservation in amino acid sequence with SPRITE-1. It also shares 29% identity and 45% conservation with the *copia* element from *Drosophila*. No elements were found to have nucleotide similarity with the LTR of SPRITE-1 further indicating that this is a member of a unique family of Ty1/*copia* type elements.

SPRITE-1 has the component retrovirus-like amino acid motifs that code for the proteins necessary for transposition. These motifs are the gag-related protein that contains a Cys-His box also known as the CCHC zinc-binding domain, a protease, an integrase, reverse transcriptase and RNase H. These motifs are ordered as they are in Ty1 and *copia*. FIG. 17 shows amino acid alignments of these conserved region from the similar retroelements previously mentioned. These motifs were similarly positioned relative to each other in these retroelements except the CCHC zinc binding domain which was more variant in position relative to the protease motif. This motif was aligned by hand whereas the alignments of the other motifs were constructed by CLUSTAL W and processed using BOXSHADE. Alignments indicate that SPRITE-1 does possess the component protein coding regions necessary for replication and transposition. The coding regions of many retroelements have shown mutations that create frameshifts or introduce stop codons thus preventing translation of functional proteins and preventing transposition. The coding region of SPRITE-1 is intact and therefore has the potential to transpose.

The number of copies of SPRITE-1 is relatively low but variable.

A survey of inbred lines developed from several different populations and other genetic stocks revealed differences in SPRITE-1 copy number. DNA was digested with *Eco*RI and southern blots hybridized with a probe representing the 5' untranslated region of SPRITE-1. This element does not have any *Eco*RI restriction sites. SPRITE-1 is found at a low copy number in most maize lines. Copy number varies from 3 as in B73 and Mo17 to 5 as in B14 and B79 (FIG. 18). The insertion of SPRITE-1 into zmet2a is only found in Mo17 and not in any other maize inbred line except A682, a line derived from Mo17 (FIG. 19). C.I. 187-2, a Mo17 parental line, does not contain SPRITE-1. This indicates that SPRITE-1 has been active recently, i.e. after the origin of the maize populations used for inbred development.

Expression of SPRITE-1

Expression was investigated by hybridizing a southern blot of cDNAs, sythesized from mRNA from different maize tissues, with a SPRITE-1 probe (FIG. 20). Expression of SRITE-1 was highest in leaf tissue. Expression was highest in leaf tissue from plants with a *MUTATOR* insertion in zmet2a and decreased CpNpG methylation. A low level of expression was observed in most tissues, but this may be due to transcription of other genes containing SPRITE-1 in a sense orientation.

SPRITE-1 does not effect zmet2a transcript processing.

During the sequencing of zmet2a cDNA, no fragments or subclones possessed SPRITE-1 sequence indicating that it is efficiently spliced from the transcript. Aberrant splicing has been observed in genes containing retroelements (Pouteau et al., *Mol. Gen. Genet.*, 228:233-239 (1991), Varagona et al., *Plant Cell*, 4:811-820 (1992), Marillonnet and Wessler, *Plant Cell*, 9:967-978 (1997), Kapitonov and Jurka, *J. Mol. Evol.*, 48:248-251 (1999)). Expression of three alleles of the *waxy* gene of maize was low due to retroelement insertions within introns (Varagona et al., *Plant Cell*, 4:811-820 (1992)). Varagona et al. (*Plant Cell*, 4:811-820 (1992)) found that although the element was spliced out of the *waxy* transcript, long-range splice site recognition was disrupted as exons upstream and downstream of the insertion site were found to be excluded in some transcripts. Further analysis of the *wxG* allele showed tissue specific differences in RNA processing with more correctly spliced transcripts in pollen than in the endosperm (Marillonnet and Wessler, *Plant Cell*, 9:967-978 (1997)).

Alternatively spliced transcripts were searched for by PCR amplification of fragments spanning several exons both upstream and downstream of the SPRITE-1 insertion site. Fragments were amplified from Mo17 seedling and immature embryo cDNA and compared to fragments amplified from B73 immature ear cDNA (FIG. 21). Amplification products were separated on an agarose gel and southern blotted. The Southern blot was hybridized to a near full length zmet2a cDNA. No differences were observed between the B73 and Mo17 products indicating that only correctly spliced fragments were detected. The blot was stripped and probed with retroelement sequences. No transcripts were amplified that contained any SPRITE-1 sequence. In

the tissues examined in this example, no aberrant transcripts were detected. Aberrant splicing products may be at such a low concentration that they are not detectable.

SPRITE-1 does not effect zmet2a expression and function.

Since SPRITE-1 is inserted into an intron of zmet2a, the effect of this insertion on zmet2a activity was investigated. HPLC data shows no methylation differences among the recombinant inbred lines with or without a SPRITE-1 insertion in zmet2a. Lines with a SPRITE-1 insertion had 18.21% ± 1.78 5-methylcytosine whereas lines without the insertion had 18.20% ± 0.24. It is probable that most transcripts are processed correctly since no changes in methylation are observed in plants with a SPRITE-1 insertion.

Regions of SPRITE-1 are hypermethylated

Portions of SPRITE-1 were examined to determine the status of cytosine methylation. Using methylation sensitive restriction enzymes, sites within 970 bp of the untranslated region (hereinafter "UTR") immediately downstream from the transcription start site was analyzed. FIG. 22 shows methylation sensitive restriction digestion patterns for Mo17 and B73. The isoschizomers *Hpa*II and *Msp*I recognize CCGG sequences and are differentially sensitive to methylation. SPRITE-1 has a single *MspI/Hpa*II site. Using the SPRITE-1 sequence from Mo17, the zmet2a insertion of SPRITE-1 would generate fragments of 5853 bp and 4625 bp. Other SPRITE-1 insertions would generate fragments of variable lengths. Southern blots show only very large fragments >20 Kb for both *Hpa*II and *Msp*I. *Msp*I does show a smaller fragment size than *Hpa*II but is much larger than the expected size for the zmet2a insertion. This indicates that this site is methylated in most SPRITE-1 copies.

Another pair of isoschizomers *Bst*NI and *Eco*RII recognize the sequence CC(A/T)GG. *BstN*I is not sensitive to methylation and *Eco*RII will not cut when the internal cytosine is methylated. *Bst*NI should generate SPRITE-1-specific fragments of 6, 54, 135, 252, and 784 bp with the UTR probe. All *Eco*RII fragments were greater than 20 Kb indicating complete methylation of these sites. *Hha*I which recognizes GCGC sites should generate SPRITE-1-specific fragments of 2884 and 257 bp and a zmet2a insertion fragment of 2965 bp. No fragments this small were

observed indicating methylation at these sites. The *Pst*I site recognized with this probe was also methylated.

EXAMPLE 2 – Cloning and Sequencing of zmet2b

A lambda library (Stratagene) constructed from Mo17 maize genomic DNA library was screened with the zmet2a methyltransferase nucleic sequences shown in FIG. 1. This screening resulted in the recovery of seven (7) independent clones. Four of these clones corresponded exactly to zmet2a nucleic acid sequence. Another type, represented by only one clone, had limited homology in non-significant regions. Two other clones were very similar to the zmet2a methyltransferase nucleic acid sequence but were definitely not identical to the zmet2a methyltransferase nucleic acid sequence. These clones defined a second gene, referred to as "zmet2b". Primer walking resulted in a partial genomic sequence of zmet2b. Primers specific to zmet2b were designed and used to amplify zmet2b cDNA (using Marathon cDNA Amplification Kit from Clontech according to the manufacturer's protocols). The RACE products were isolated and cloned into p-GEMT-Easy (Promega). Sequence of the RACE products generated a partial cDNA sequence for the 3' end of the gene (see FIG. 23). A partial amino acid sequence encoded by this cDNA sequence is shown in FIG. 24. A comparison of a portion of the amino acid sequences for zmet2a and zmet2b is shown in FIG. 25.

All references cited herein are hereby incorporated by reference.

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof. It is intended that all such variations within the scope and spirit of the appended claims be embraced thereby.

Changes can be made to the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention as defined in the following claims.

WHAT IS CLAIMED IS:

1. An isolated and purified *Zea mays* zmet2a methyltransferase nucleic acid sequence.

2. The nucleic acid sequence of claim 1 wherein the nucleic acid sequence hybridizes to the nucleic acid sequence of FIG. 1A under stringent conditions.

3. A zmet2a methyltransferase comprising the amino acid sequence shown in FIG. 2A.

4. The nucleic acid sequence of claim 1 wherein the nucleic acid sequence hybridizes to the nucleic acid sequenceof FIG. 1B under stringent conditions.

5. A zmet2a methyltransferase comprising the amino acid sequence shown in FIG. 2B.

6. A recombinant expression cassette comprising the isolated and purified nucleic acid sequence of claim 1, a promoter sequence and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the nucleic acid sequence and the nucleic acid sequence is operably linked to the polyadenylation signal sequence.

7. The recombinant expression cassette of claim 6 wherein the promoter sequence is a constitutive or a tissue specific promoter sequence.

8. A recombinant expression cassette comprising a heterologous nucleic acid sequence, a promoter sequence from the nucleic acid sequence of claim 1 and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the heterologous nucleic acid sequence and the heterologous nucleic acid sequence is operably linked to the polyadenylation signal sequence.

9. A bacterial cell comprising the recombinant expression cassette of claims 6 or 8.

10. The bacterial cell of claim 9 wherein the bacterial cell is selected from the group consisting of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

11. A transgenic plant cell comprising the recombinant expression cassette of claims 6 or 8.

12. The transgenic plant cell of claim 11 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

13. A transgenic plant comprising the recombinant expression cassette of claims 6 or 8.

14. The transgenic plant of claim 13 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

15. The transgenic plant of claim 13 wherein transgenic plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

16. Seed from the transgenic plant of claim 13.

17. A process for methylating a target gene in a plant, the process comprising the steps of:

transforming a plant with a recombinant expression cassette comprising a tissue specific promoter and the nucleic acid sequence of claim 1, the tissue specific promoter being operably linked to the nucleic acid sequence, wherein the tissue-specific promoter directs expression of the nucleic acid sequence, and the expression of the nucleic acid

sequence produces zmet2a methyltransferase in sufficient quantities in the area containing the target gene to allow for methylation of the target gene.

18. The process of claim 17 wherein the plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*. *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

19. An isolated and purified *Zea mays* zmet2b methyltransferase nucleic acid sequence.

20. An isolated and purified *Zea mays* zmet2b methyltransferase nucleic acid sequence which hybridizes to FIG. 23 under stringent conditions.

21. A recombinant expression cassette comprising the isolated and purified nucleic acid sequence of claim 19, a promoter sequence and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the nucleic acid sequence and the nucleic acid sequence is operably linked to the polyadenylation signal sequence.

22. The recombinant expression cassette of claim 21 wherein the promoter sequence is a constitutive or a tissue specific promoter sequence.

23. A recombinant expression cassette comprising a heterologous nucleic acid sequence, a promoter sequence from the nucleic acid sequence of claim 19 and a polyadenylation signal sequence, wherein the promoter sequence is operably linked to the heterologous nucleic acid sequence and the heterologous nucleic acid sequence is operably linked to the polyadenylation signal sequence.

24. A bacterial cell comprising the recombinant expression cassette of claims 21 or 23.

25. The bacterial cell of claim 24 wherein the bacterial cell is selected from the group consisting of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

26. A transgenic plant cell comprising the recombinant expression cassette of claims 21 or 23.

27. The transgenic plant cell of claim 26 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

28. A transgenic plant comprising the recombinant expression cassette of claims 21 or 23.

29. The transgenic plant of claim 28 wherein the promoter sequence and the polyadenylation signal sequence is from Cauliflower Mosaic Virus 35S gene.

30. The transgenic plant of claim 28 wherein transgenic plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*, *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, and *Brassica napus*.

31. Seed from the transgenic plant of claim 28.

32. A process for methylating a target gene in a plant, the process comprising the steps of:

transforming a plant with a recombinant expression cassette comprising a tissue specific promoter and the nucleic acid sequence of claim 19, the tissue specific promoter being operably linked to the nucleic acid sequence. wherein the tissue-specific promoter directs expression of the nucleic acid sequence, and the expression of the nucleic acid sequence produces zmet2b methyltransferase in sufficient quantities in the area containing the target gene to allow for methylation of the target gene.

33. The process of claim 32 wherein the plant is *Zea mays*, *Oryza sativa*, *Secale cereale*, *Triticum aestivum*, *Daucus carota*, *Brassica oleracea*, *Cucumis melo*, *Cucumis sativus*. *Latuca sativa*, *Solanum tubersoum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*. and *Brassica napus*.

FIG. 1A 2736 bp

1 ATGGCGCCGA GCTCCCCGTC ACCCGCCGCG CCTACACGCG TCTCTGGGCG
51 GAAGCGCGCC GCCAAGGCCG AGGAGATCCA CCAGAACAAG GAGGAGGAGG
101 AGGAGGTCGC GGCGGCGTCC TCCGCCAAGC GCAGCCGCAA GGCGGCATCT
151 TCCGGGAAGA AGCCCAAGTC GCCCCCCAAG CAGGCCAAGC CGGGGAGGAA
201 GAAGAAGGGG GATGCCGAGA TGAAGGAGCC CGTGGAGGAC GACGTGTGCG
251 CCGAGGAGCC CGACGAGGAG GAGTTGGCCA TGGGCGAGGA GGAGGCCGAG
301 GAGCAGGCCA TGCAGGAGGA GGTGGTTGCG GTCGCGGCGG GGTCACCCGG
351 GAAGAAGAGG GTGGGGAGAA GGAACGCCGC CGCCGCCGCT GGCGACCACG
401 AGCCGGAGTT CATCGGCAGC CCTGTTGCCG CGGACGAGGC GCGCTGCAAC
451 TGGCCCAAGC GCTACGGCCG CAGCACTGCC GCAAAGAAAC CGGATGAGGA
501 GGAAGAGCTC AAGGCCAGAT GTCACTACCG GAGCGCTAAG GTGGACAACG
551 TCGTCTACTG CCTCGGGGAT GACGTCTATG TCAAGGCTGG AGAAAACGAG
601 GCAGATTACA TTGGCCGCAT TACTGAATTT TTTGAGGGGA CTGACCAGTG
651 TCACTATTTT ACTTGCCGTT GGTTCTTCCG AGCAGAGGAC ACGGTTATCA
701 ATTCTTTGGT GTCCATAAGT GTGGATGGCC ACAAGCATGA CCCTAGACGT
751 GTTTTTCTTT CTGAGGAAAA GAACGACAAT GTGCTTGATT GCATTATCTC
801 CAAGGTCAAG ATAGTCCATG TTGATCCAAA TATGGATCCA AAAGCCAAGG
851 CTCAGCTGAT AGAGAGTTGC GACCTATACT ATGACATGTC TTACTCTGTT
901 GCATATTCTA CATTTGCTAA TATCTCGTCT GAAAATGGGC AGTCAGGCAG
951 TGATACCGCT TCGGGTATTT CTTCTGATGA TGTGGATCTG GAGACGTCAT
1001 CTAGTATGCC AACGAGGACA GCAACCCTTC TTGATCTGTA TTCTGGCTGT
1051 GGGGGCATGT CTACTGGTCT TTGCTTGGGT GCAGCTCTTT CTGGCTTGAA
1101 ACTTGAAACT CGATGGGCTG TTGATTTCAA CAGTTTTGCG TGCCAAAGTT
1151 TAAAATATAA TCATCCACAG ACTGAGGTGC GAAATGAGAA AGCCGATGAG
1201 TTTCTTGCCC TCCTTAAGGA ATGGGCAGTT CTATGCAAAA AATATGTCCA
1251 AGATGTGGAT TCAAATTTAG CAAGCTCAGA GGATCAAGCG GATGAAGACA
1301 GCCCTCTTGA CAAGGACGAA TTTGTTGTAG AGAAGCTTGT CGGGATATGT
1351 TATGGTGGCA GTGACAGGGA AAATGGCATC TATTTTAAGG TCCAGTGGGA
1401 AGGATACGGC CCTGAGGAGG ATACATGGGA ACCGATTGAT AACTTGAGTG
1451 ACTGCCCGCA GAAAATTAGA GAATTTGTAC AAGAAGGGCA CAAAAGAAAG
1501 ATTCTCCCAC TGCCTGGTGA TGTTGATGTC ATTTGTGGAG GCCCACCATG
1551 CCAAGGTATC AGTGGGTTTA ATCGGTACAG AAACCGTGAT GAGCCACTCA
1601 AAGATGAGAA AAACAAACAA ATGGTGACTT TCATGGATAT TGTGGCGTAC
1651 TTGAAGCCCA AGTATGTTCT CATGGAAAAT GTGGTGGACA TACTCAAATT
1701 TGCGGATGGT TACCTAGGAA AATATGCTTT GAGCTGCCTT GTTGCTATGA
1751 AGTACCAAGC GCGGCTTGGA ATGATGGTGG CTGGTTGCTA TGGTCTGCCA
1801 CAGTTCAGGA TGCGTGTGTT CCTCTGGGGT GCTCTTTCTT CCATGGTGCT
1851 CCCTAAGTAT CCTCTGCCCA CCTATGATGT TGTAGTACGT GGAGGAGCCC
1901 CTAATGCCTT TTCGCAATGT ATGGTTGCAT ATGACGAGAC ACAAAAACCA
1951 TCCCTGAAAA AAGCCTTGCT TCTTGGCGAT GCAATTTCAG ATTTACCAAA
2001 GGTTCAAAAT CACCAGCCTA ACGATGTGAT GGAGTATGGT GGTTCCCCCA
2051 AGACCGAATT CCAGCGCTAC ATTCGACTCA GTCGTAAAGA CATGTTGGAT
2101 TGGTCCTTCG GTGAGGGGGC TGGTCCAGAT GAAGGCAAGC TCTTGGATCA
2151 CCAGCCTTTA CGGCTTAACA ACGATGATTA TGAGCGGGTT CAACAGATTC
2201 CTGTCAAGAA GGGAGCCAAC TTCCGCGACC TAAAGGGCGT GAGGGTTGGA
2251 GCAAACAATA TTGTTGAGTG GGATCCAGAA ATCGAGCGTG TGAAACTTTC
2301 ATCTGGAAA CCACTGGTTC CTGACTATGC AATGTCATTC ATCAAGGGCA
2351 AATCACTCAA GCCGTTTGGG CGCCTGTGGT GGGACGAGAC AGTTCCTACA
2401 GTTGTAACCA GAGCAGAGCC TCACAACCAG GTTATAATTC ATCCGACTCA
2451 AGCAAGGGTC CTCACTATCC GGGAGAACGC AAGGTTACAG GGCTTCCCCG
2501 ATTACTACCG ATTGTTTGGC CCGATCAAGG AGAAGTACAT TCAAGTCGGG
2551 AACGCAGTGG CTGTCCCTGT TGCCCGGGCA CTGGGCTACT GTCTGGGGCA
2601 AGCCTACCTG GGTGAATCTG AGGGGAGTGA CCCTCTGTAC CAGCTGCCTC
2651 CAAGTTTCAC CTCTGTTGGA GGACGCACTG CGGGGCAGGC GAGGGCCTCT
2701 CCTGTTGGCA CCCCTGCAGG GGAGGTAGTT GAGCAG

FIG. 1B

```
   1  AGAGCAGCAG CAGCTACCGC AGCCCCTGCC ATGGCGCCGA GCTCCCCGTC
  51  ACCCGCCGCG CCTACACGCG TCTCTGGGCG GAAGCGCGCC GCCAAGGCCG
 101  AGGAGATCCA CCAGAACAAG GAGGAGGAGG AGGAGGTCGC GGCGGCGTCC
 151  TCCGCCAAGC GCAGCCGCAA GGCGGCATCT TCCGGGAAGA AGCCCAAGTC
 201  GCCCCCCAAG CAGGCCAAGC CGGGGAGGAA GAAGAAGGGG GATGCCGAGA
 251  TGAAGGAGCC CGTGGAGGAC GACGTGTGCG CCGAGGAGCC CGACGAGGAG
 301  GAGTTGGCCA TGGGCCAGGA GGAGGCCGAG GAGCAGGCCA TGCAGGAGGA
 351  GGTGGTTGCG GTCGCGGCGG GGTCACCCGG GAAGAAGAGG GTGGGGAGAA
 401  GGAACGCCGC CGCCGCCGCT GGCGACCACG AGCCGGAGTT CATCGGCAGC
 451  CCTGTTGCCG CGGACGAGGC GCGCAGCAAC TGGCCCAAGC GCTACGGCCG
 501  CAGCACTGCC GCAAAGAAAC CGGATGAGGA GGAAGAGCTC AAGGCCAGAT
 551  GTCACTACCG GAGCGCTAAG GTGGACAACG TCGTCTACTG CCTCGGGGAT
 601  GACGTCTATG TCAAGGCTGG AGAAAACGAG GCAGATTACA TTGGCCGCAT
 651  TACTGAATTT TTTGAGGGGA CTGACCAGTG TCACTATTTT ACTTGCCGTT
 701  GGTTCTTCCG AGCAGAGGAC ACGGTTATCA ATTCTTTGGT GTCCATAAGT
 751  GTGGATGGCC ACAAGCATGA CCCTAGACGT GTTTTTCTTT CTGAGGAAAA
 801  GAACGACAAT GTGCTTGATT GCATTATCTC CAAGGTCAAG ATAGTCCATG
 851  TTGATCCAAA TATGGATCCA AAAGCCAAGG CTCAGCTGAT AGAGAGTTGC
 901  GACCTATACT ATGACATGTC TTACTCTGTT GCATATTCTA CATTTGCTAA
 951  TATCTCGTCT GAAAATGGGC AGTCAGGCAG TGATACCGCT TCGGGTATTT
1001  CTTCTGATGA TGTGGATCTG GAGACGTCAT CTAGTATGCC AACGAGGACA
1051  GCAACCCTTC TTGATCTGTA TTCTGGCTGT GGGGGCATGT CTACTGGTCT
1101  TTGCTTGGGT GCAGCTCTTT CTGGCTTGAA ACTTGAAACT CGATGGGCTG
1151  TTGATTTCAA CAGTTTTGCG TGCCAAAGTT TAAAATATAA TCATCCACAG
1201  ACTGAGGTGC GAAATGAGAA AGCCGATGAG TTTCTTGCCC TCCTTAAGGA
1251  ATGGGCAGTT CTATGCAAAA AATATGTCCA AGATGTGGAT TCAAATTTAG
1301  CAAGCTCAGA GGATCAAGCG GATGAAGACA GCCCTCTTGA CAAGGACGAA
1351  TTTGTTGTAG AGAAGCTTGT CGGGATATGT TATGGTGGCA GTGACAGGGA
1401  AAATGGCATC TATTTTAAGG TCCAGTGGGA AGGATACGGC CCTGAGGAGG
1451  ATACATGGGA ACCGATTGAT AACTTGAGTG ACTGCCCGCA GAAAATTAGA
1501  GAATTTGTAC AAGAAGGGCA CAAAAGAAAG ATTCTCCCAC TGCCTGGTGA
1551  TGTTGATGTC ATTTGTGGAG GCCCACCATG CCAAGGTATC AGTGGGTTTA
1601  ATCGGTACAG AAACCGTGAT GAGCCACTCA AAGATGAGAA AAACAAACAA
1651  ATGGTGACTT TCATGGATAT TGTGGCGTAC TTGAAGCCCA AGTATGTTCT
1701  CATGGAAAAT GTGGTGGACA TACTCAAATT TGCGGATGGT TACCTAGGAA
1751  AATATGCTTT GAGCTGCCTT GTTGCTATGA AGTACCAAGC GCGGCTTGGA
1801  ATGATGGTGG CTGGTTGCTA TGGTCTGCCA CAGTTCAGGA TGCGTGTGTT
1851  CCTCTGGGGT GCTCTTTCTT CCATGGTGCT CCCTAAGTAT CCTCTGCCCA
1901  CCTATGATGT TGTAGTACGT GGAGGAGCCC CTAATGCCTT TTCGCAATGT
1951  ATGGTTGCAT ATGACGAGAC ACAAAAACCA TCCCTGAAAA AAGCCTTGCT
2001  TCTTGGCGAT GCAATTTCAG ATTTACCAAA GGTTCAAAAT CACCAGCCTA
2051  ACGATGTGAT GGAGTATGGT GGTTCCCCCA AGACCGAATT CCAGCGCTAC
2101  ATTCGACTCA GTCGTAAAGA CATGTTGGAT TGGTCCTTCG GTGAGGGGGC
2151  TGGTCCAGAT GAAGGCAAGC TCTTGGATCA CCAGCCTTTA CGGCTTAACA
2201  ACGATGATTA TGAGCGGGTT CAACAGATTC CTGTCAAGAA GGGAGCCAAC
2251  TTCCGCGACC TAAAGGGCGT GAGGGTTGGA GCAAACAATA TTGTTGAGTG
2301  GGATCCAGAA ATCGAGCGTG TGAAACTTTC ATCTGGGAAA CCACTGGTTC
2351  CTGACTATGC AATGTCATTC ATCAAGGGCA AATCACTCAA GCCGTTTGGG
2401  CGCCTGTGGT GGGACGAGAC AGTTCCTACA GTTGTAACCA GAGCAGAGCC
2451  TCACAACCAG GTTATAATTC ATCCGACTCA AGCAAGGGTC CTCACTATCC
2501  GGGAGAACGC AAGGTTACAG GGCTTCCCCG ATTACTACCG ATTGTTTGGC
2551  CCGATCAAGG AGAAGTACAT TCAAGTCGGG AACGCAGTGG CTGTCCCTGT
2601  TGCCCGGGCA CTGGGCTACT GTCTGGGGCA AGCCTACCTG GGTGAATCTG
2651  AGGGGAGTGA CCCTCTGTAC CAGCTGCCTC CAAGTTTCAC CTCTGTTGGA
2701  GGACGCACTG CGGGGCAGGC GAGGGCCTCT CCTGTTGGCA CCCCTGCAGG
2751  GGAGGTAGTT GAGCAGTAAA AGGATGACAG ATCTGAGCTG AGCTGG
```

FIG. 2A **912 amino acids**

```
  1  MAPSSPSPAA PTRVSGRKRA AKAEEIHQNK EEEEEVAAAS SAKRSRKAAS
 51  SGKKPKSPPK QAKPGRKKKG DAEMKEPVED DVCAEEPDEE ELAMGEEEAE
101  EQAMQEEVVA VAAGSPGKKR VGRRNAAAAA GDHEPEFIGS PVAADEARSN
151  WPKRYGRSTA AKKPDEEEEL KARCHYRSAK VDNVVYCLGD DVYVKAGENE
201  ADYIGRITEF FEGTDQCHYF TCRWFFRAED TVINSLVSIS VDGHKHDPRR
251  VFLSEEKNDN VLDCIISKVK IVHVDPNMDP KAKAQLIESC DLYYDMSYSV
301  AYSTFANISS ENGQSGSDTA SGISSDDVDL ETSSSMPTRT ATLLDLYSGC
351  GGMSTGLCLG AALSGLKLET RWAVDFNSFA CQSLKYNHPQ TEVRNEKADE
401  FLALLKEWAV LCKKYVQDVD SNLASSEDQA DEDSPLDKDE FVVEKLVGIC
451  YGGSDRENGI YFKVQWEGYG PEEDTWEPID NLSDCPQKIR EFVQEGHKRK
501  ILPLPGDVDV ICGGPPCQGI SGFNRYRNRD EPLKDEKNKQ MVTFMDIVAY
551  LKPKYVLMEN VVDILKFADG YLGKYALSCL VAMKYQARLG MMVAGCYGLP
601  QFRMRVFLWG ALSSMVLPKY PLPTYDVVVR GGAPNAFSQC MVAYDETQKP
651  SLKKALLLGD AISDLPKVQN HQPNDVMEYG GSPKTEFQRY IRLSRKDMLD
701  WSFGEGAGPD EGKLLDHQPL RLNNDDYERV QQIPVKKGAN FRDLKGVRVG
751  ANNIVEWDPE IERVKLSSGK PLVPDYAMSF IKGKSLKPFG RLWWDETVPT
801  VVTRAEPHNQ VIIHPTQARV LTIRENARLQ GFPDYYRLFG PIKEKYIQVG
851  NAVAVPVARA LGYCLGQAYL GESEGSDPLY QLPPSFTSVG GRTAGQARAS
901  PVGTPAGEVV EQ
```

FIG. 2B

RAAAATAAPAMAPSSPSPAAPTRVSGRKRAAKAEEIHQNKEEEEEVAAAS
SAKRSRKAASSGKKPKSPPKQAKPGRKKKGDAEMKEPVEDDVCAEEPDEE
ELAMGEEEAEEQAMQEEVVAVAAGSPGKKRVGRRNAAAAAGDHEPEFIGS
PVAADEARSNWPKRYGRSTAAKKPDEEEELKARCHYRSAKVDNVVYCLGD
DVYVKAGENEADYIGRITEFFEGTDQCHYFTCRWFFRAEDTVINSLVSIS
VDGHKHDPRRVFLSEEKNDNVLDCIISKVKIVHVDPNMDPKAKAQLIESC
DLYYDMSYSVAYSTFANISSENGQSGSDTASGISSDDVDLETSSSMPTRT
ATLLDLYSGCGGMSTGLCLGAALSGLKLETRWAVDFNSFACQSLKYNHPQ
TEVRNEKADEFLALLKEWAVLCKKYVQDVDSNLASSEDQADEDSPLDKDE
FVVEKLVGICYGGSDRENGIYFKVQWEGYGPEEDTWEPIDNLSDCPQKIR
EFVQEGHKRKILPLPGDVDVICGGPPCQGISGFNRYRNRDEPLKDEKNKQ
MVTFMDIVAYLKPKYVLMENVVDILKFADGYLGKYALSCLVAMKYQARLG
MMVAGCYGLPQFRMRVFLWGALSSMVLPKYPLPTYDVVVRGGAPNAFSQC
MVAYDETQKPSLKKALLLGDAISDLPKVQNHQPNDVMEYGGSPKTEFQRY
IRLSRKDMLDWSFGEGAGPDEGKLLDHQPLRLNNDDYERVQQIPVKKGAN
FRDLKGVRVGANNIVEWDPEIERVKLSSGKPLVPDYAMSFIKGKSLKPFG
RLWWDETVPTVVTRAEPHNQVIIHPTQARVLTIRENARLQGFPDYYRLFG
PIKEKYIQVGNAVAVPVARALGYCLGQAYLGESEGSDPLYQLPPSFTSVG
GRTAGQARASPVGTPAGEVVEQ*KDDRSELSW

FIG. 3

Primer	Sequence 5' - 3'
1F	TGGTTGCTATGGTCTGCCACAGTTCAG
1R	CCAGCTCAGCTCAGATCTGTCATCCTTT
Seq2FN	CGAAAGCTAATCTACACAAACAGC
Seq2RN	GATCCTCTGAGCTTGCTAAATTTG
3R	CTCATCTTGGAGTGGCTCATCAC
S3F	GAGCACATGAGGGAGAGTGTTG
S3R	TCTCTAATTTTCTGCGGGCAG
4F	CCTCTGCCCACCTATGATGTTGTA
5F	TAAAGGGCGTGAGGGTTGGA
7F	TCACATTTGTCATGGCAGGTTATC
8eF	CTGAGGAAAAGAACGACAATGTGC
8eR	GCAATCAAGCACATTGTCGTTCTTTTCCTC
9eF	GAAGAAGAGGGTGGGGAGAAGGAACG
9eR	TTCTTTGCGGCAGTGCTGCG
11iF	GTATTGAATTGATTCTCAACTAGTGCAC
11iR	CAGGCTCAACGGCGATG
12iF	TATGCTTCATCACATAGACCCAAGTC
12iR	GATAGACCTAATGCCAAATGAGATTAAG
13iF	GCGATCTTCAGTCTCCACCATC
13iR	GAAGACGTGCCTCCATGTTTCATC
14F	GTTGGTTCTTCCGAGCAGAGG
14R	GACTGCCACATATCTTATTAATCGC
15F	GCATGTGTCAGCAATTGCTTACATTC
15R	CCTCTGCTCGGAAGAACCAAC
16F	CTGTTCGGAGATTCATGCATGATG
16R	GGAGAACAGAATGGTTGATTCAATGG
17F	GCACTTCACTCTCCTGGCAAACC
17R	CGGTACGCTGCTGCTGCTCTC
18F	CCATAGCATCTCACATATCGCAAGG
18R	GGAAAGAAGGCAGTTAGTTGTAAATGGG
MU	AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC
RaceRT	CTACAACATCATAGTTGGGCAGAGG
AP2 marathon	ACTCACTATAGGGCTCGAGCGGC
T7	TAATACGACTCACTATAGGG
Sp6	GATTTAGGTGACACTATAG
M13F	GTTTTCCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC

FIG. 4

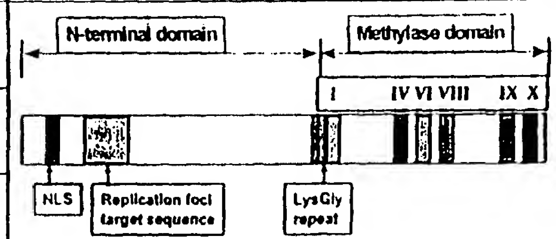
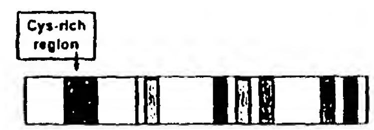
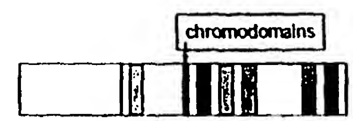
Gene Name	Organism	Function	General Structure
DNMT1/Dnmt1	human/mouse	maintenance	
MET1	*Arabidopsis*	maintenance	
Zmet1	maize	putative maintenance	
DNMT3/Dnmt3	human/mouse	*de novo*	
Zmet3	maize	putative *de novo*	
DRM	*Arabidopsis*	putative *de novo*	
CMT1	*Arabidopsis*	undetermined (putative CpNpG)	
Zmet2a	maize	CpNpG (maintenance and/or *de novo*)	

Figure 5

```
                          HhaI                                 MseI
tacatcaataaaataaggggcgccaacgcaattgtcccttGtttttctaacttaaagttcaagcggcaatgtcg base pairs
atgtagttattttattccccgcggttgcgttaacagggaacaaaaagattgaatttcaagttcgccgttacagc 1 to 75
                                                       MseI        MseI
catctgatgtatgaatatcaatttgtaagtactaaacatagtcttaattccaaaattaattacacaacaaagact base pairs
gtagactacatacttatagttaaacattcatgatttgtatcagaattaaggttttaattaatgtgttgtttctga 76 to 150

                                MseI                                 HinfI
aaattgtaagcaaacctttcaagtctaattaattcataattacaaatgttattgtaacatcatgttaccgaatca base pairs
tttaacattcgtttggaaagttcagattaattaagtattaatgtttacaataacattgtagtacaatggcttagt 151 to 225

          ScrFI
        EcoRII                                   MseI
taaactaaccaggttcccatgtgtaattagtttataattatattatatttaatatttgtaactaattgatgtga base pairs
atttgattggtccaagggtacacattaatcaaaatattaatataatataaattataaacattgattaactacact 226 to 300
          BstNI

            MseI      MseI                            MseI
cagtactaaaattaagcctcttaagccaaaaaatccacatatttagatttaaaatttgaaaacagacgtatcgg base pairs
gtcatgattttaattcggagaattcggttttttaggtgtataaatctaaattttaaacttttgtctgcatagcc 301 to 375

                                                   HaeIII
ctagaagagccctgtcactgtcagctaatcaattacaagaagtggcccatactagttccatcaccagtccagtag base pairs
gatcttctcgggacagtgacagtcgattagttaatgttcttcaccgggtatgatcaaggtagtggtcaggtcatc 376 to 450

                    PvuII                    HaeIII HaeIII  HhaI HhaI  HpaII
tccaccaccccaccctacagctgggtcatctggcacgggtggagggggccaacggccaaaagcgccgcgcacttcc base pairs
aggtggtggggtgggatgtcgacccagtagaccgtgcccacctccccggttgccggttttcgcggcgcgtgaagg 451 to 525
                                                                       MspI

                                                                     ApaI
                 HinfI                                          PstI EcoO109I
ggcgggcaccctCgcggagtcgcgggtgacagcgaaatttcaaatccataccctcccgctgcagacgggccccac base pairs
ccgcccgtgggagcgcctcagcgcccactgtcgctttaaagtttaggtatgggagggcgacgtctgcccggggtg 526 to 600
                                                                     HaeIII

                               TaqI
gccgtcaaaatttggacgctcccgctccctcgatcttttgggtttcgttttcccagttcccacccctctcttccac base pairs
cggcagttttaaacctgcgagggcgagggagctagaaaacccaaagcaaaagggtcaagggtgggagagaaggtg 601 to 675
                                Sau3AI

                       Sau3AI   TaqI TaqI                     HinfI
cctgccctgtttccagatttgaccgatccccttcgattcgatttctacacccacggtgtccagactccagagcac base pairs
ggacgggacaaaggtctaaactggctaggggaagctaagctaaagatgtgggtgccacaggtctgaggtctcgtg 676 to 750
                                    HinfI
         ScrFI
 17F    EcoRII
tcactctcctggcaaacccctttcgtcttcccaaccctagagagcagcagcagctaccgcagcccctgccatggc base pairs
agtgagaggaccgtttggggaaagcagaagggttgggatctctcgtcgtcgtcgatggcgtcggggacggtaccg 751 to 825
         BstNI                                        17R
 HhaI    SacI          HhaI                      HhaI       HaeIII Sau3AI
gccgagctccccgtcacccgccgcgcctacacgcgtctctgggcggaagcgcgccgccaaggccgaggagatcca base pairs
cggctcgaggggcagtgggcggcgcggatgtgcgcagagacccgccttcgcgcggcggttccggctcctctaggt 826 to 900

                                                                      ScrFI
                                                 HhaI                HpaII
ccagaacaaggaggaggaggaggaggtcgcggcggcgtcctccgccaagcgcagccgcaaggcggcatcttccgg base pairs
ggtcttgttcctcctcctcctcctccagcgccgccgcaggaggcggttcgcgtcggcgttccgccgtagaaggcc 901 to 975
                                                                    MspI

                                    MspI
                                HaeIII ScrFI
gaagaagcccaagtcgccccccaagcaggccaagccggggaggaagaagaaggggggatgccgagatgaaggagcc base pairs
cttcttcgggttcagcggggggttcgtccggttcggcccctccttcttcttcccccctacggctctacttcctcgg 976 to 1050
                                    HpaII
```

FIG. 5

Continued

```
                     HhaI                          HaeIII                HaeIII
cgtggaggacgacgtgtgcgccgaggagcccgacgaggaggagttggccatgggcgaggaggaggccgaggagca base pairs
gcacctcctgctgcacacgcggctcctcgggctgctcctcctcaaccggtacccgctcctcctccggctcctcgt 1051 to 1125


                                             MspI
 HaeIII                                      HpaII        9eF
ggccatgcaggaggaggtggttgcggtcgcggcggggtcacccgggaagaagagggtggggagaaggaacgccgc base pairs
ccggtacgtcctcctccaccaacgccagcgccgccccagtgggcccttcttctcccacccctcttccttgcggcg 1126 to 1200
                                             ScrFI
                                              SmaI


                        HpaII                                     HhaI       HaeIII
cgccgccgctggcgaccacgagccggagttcatcggcagccctgttgccgcggacgaggcgcgcagcaactggcc base pairs
gcggcggcgaccgctggtgctcggcctcaagtagccgtcgggacaacggcgcctgctccgcgcgtcgttgaccgg 1201 to 1275
                        MspI

      HhaI   HaeIII
caaagcgctacggccgcagcacttgccgcaaaagaagtacattattttctcccagctctggttttgatttgacca base pairs
gtttcgcgatgccggcgtcgtgaacggcgttttcttcatgtaataaaagagggtcgagaccaaaactaaactggt 1276 to 1350
                    9eR

                                                                         HpaII
gattttactccatgtctgttagtacttgcgagctgagcaatctgctatttgctgatttattgtgcgtgcagacc base pairs
ctaaaatgaggtacagacaatcatgaacgctcgactcgttagacgataaacgactaaataacacgcacgtctgg 1351 to 1425
                                                                         MspI


                   SacI HaeIII         HpaII  HhaI
ggatgaggaggaagagctcaaggccagatgtcactaccggagcgctaaggtggacaacgtcgtctactgcctcgg base pairs
cctactcctccttctcgagttccggtctacagtgatggcctcgcgattccacctgttgcagcagatgacggagcc 1426 to 1500
                                       MspI
                    EcoO109I
ggatgacgtctatgtcaaggtccttgttcatcgctttctgttgcttctgctctcatttatgatgtgcatatgtgt base pairs
cctactgcagatacagttccaggaacaagtagcgaaagacaacgaagacgagagtaaatactacacgtatacaca 1501 to 1575
                    AvaII

   MseI                                  HinfI                           HpaII
ttgttaaggaagcaagaattgcttgattttgttgccgactcgcatttccgtgacgagttctgcgtatggtcacc base pairs
aacaattccttcgttcttaacgaactaaaacaacggctgagcgtaaaggcactgctcaagacgcataccagtgg 1576 to 1650
                                                                         MspI


                                                                 ScrFI
                                                            TaqI BstNI     Sau3AI
ggtacgtggcactgatacacaacgtggtatgctggaagtctggtagtatatttgcatcgaccaggaggtccaga base pairs
ccatgcaccgtgactatgtgttgcaccatacgaccttcagaccatcatataaacgtagctggtcctccaggtct 1651 to 1725
                                                               EcoRII AvaII


 ClaI                                       16iF  HinfI
tcgatatgtgcggtatagtgcttatttgattgcacccctgttcggagattcatgcatgatggcgtgtttagatgac base pairs
agctatacacgccatatcacgaataaactaacgtgggacaagcctctaagtacgtactaccgcacaaatctactg 1726 to 1800
 TaqI

                     ScrFI                 BstNI
               PvuII EcoRII PvuII      HaeIII    HpaII               HhaI   HinfI
gcctcccagacagctgcctgccaggcagctgattctggcccaggcgtccggaatggtgaagttgcgctggcaaga base pairs
cggagggtctgtcgacggacggtccgtcgactaagaccgggtccgcaggccttaccacttcaacgcgaccgttct 1801 to 1875
                     BstNI    HinfI    EcoRII   MspI
                                           ScrFI


                         ScrFI
      HaeIII           EcoRII
ttctcaggccacctaccaaatatgccctggagcatattgcatgcttcttttttgttctctttccttctatattt base pairs
aagagtccggtggatggtttatacgggacctcgtataacgtacgaagaaaaaacaagagaaaggaagatataaa 1876 to 1950
                         BstNI


atctcattgttagtgaagtttcacattgcacgtgtcatggaatatttactttcaaatcaacgaggagatgctagc base pairs
tagagtaacaatcacttcaaagtgtaacgtgcacagtaccttataaatgaaagtttagttgctcctctacgatcg 1951 to 2025
```

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FIG. 5

Continued

```
                                                EcoRV
attgaggtgtgtgataattattacatactagaagatatcgtgcatgttgccattgggattgcgaagaatgtggaa base pairs
taactccacacactattaatatgtatgatcttctatagcacgtacaacggtaaccctaacgcttcttacacctt 2026 to 2100

                         MseI      MseI
atgatgttggtagcttgtattaagaggttaacagttagtgggatgacatgactattagttagagatgatgtggat base pairs
tactacaaccatcgaacataattctccaattgtcaatcaccctactgtactgataatcaatctctactacaccta 2101 to 2175

agtaagtgggatatgatgtagatgacttgtgtgttgagacagaactataacatggagttggaaatgggagcagca base pairs
tcattcaccctatactacatctactgaacacacaactctgtcttgatattgtacctcaacctttaccctcgtcgt 2176 to 2250

                                                                      MseI
tggtcaaacataccctaaatgcctgtctctacacaatgtggtgattggtgtatagtctggtgttaaaagctggat base pairs
accagtttgtatgggatttacggacagagatgtgttacaccactaaccacatatcagaccacaattttcgaccta 2251 to 2325

                                                                           ScrFI
     HinfI                             MseI        XbaI                  EcoRII
actttgattctgttgaagattgtcacacccgaatttaaggacaaatctagatacatctcatatgtgcaccaggat base pairs
tgaaactaagacaacttctaacagtgtgggcttaaattcctgtttagatctatgtagagtatacacgtggtccta 2326 to 2400
                                                                           BstNI

agtgtatagataccaatgtcataatctttattacacgacgataatgtcttacaaaatatctggtgttacaagatg base pairs
tcacatatctatggttacagtattagaaataatgtgctgctattacagaatgttttatagaccacaatgttctac 2401 to 2475

                    MseI                   MseI
cacctttcaacatgtttaatgctgcaaactgttttaattaaacagaatgcagtgttttgaacaaaaaaatgctgc base pairs
gtggaaagttgtacaaattacgacgtttgacaaaattaatttgtcttacgtcacaaaacttgtttttttacgacg 2476 to 2550
                                             MseI

                                15iF                        Sau3AI     HinfI MseI
tttatcctgcatcttgttttgcatgtgtcagcaattgcttacattccattatgatctctgagattctttaaattt base pairs
aaataggacgtagaacaaaacgtacacagtcgttaacgaatgtaaggtaatactagagactctaagaaatttaaa 2551 to 2625

ctagcatgatgaaagtatttactaattcaactgaacacaaacattgtttgaatgaacaaggcaacacggatgctt base pairs
gatcgtactactttcataaatgattaagttgacttgtgtttgtaacaaacttacttgttccgttgtgcctacgaa 2626 to 2700

                                                                          MseI
ggaataatggttgtgtataatatcacttagtggtttgctctcacaccacatctttcatgggttctttaataata base pairs
ccttattaccaacacatattatagtgaatcaccaaaacgagagtgtggtgtagaaagtacccaagaaattattat 2701 to 2775

              MseI                                                            HaeIII
gttactgactttaagtttcttattccttttgtctatcttagctggagaaaacgaggcagattacattggccgc base pairs
caatgactgaaattcaaagaataaggaaaaacagatagaatcgacctcttttgctccgtctaatgtaaccggcg 2776 to 2850

                                                            14eF
attactgaatttttgaggggactgaccagtgtcactattttacttgccgttggttcttccgagcagaggacacg base pairs
taatgacttaaaaactcccctgactggtcacagtgataaaatgaacggcaaccaagaaggctcgtctcctgtgc 2851 to 2925
                                                            15eR

gtgtgtatttagtattttgtcattctatgcatgtgtggattttctggaatgtggaaaacatacagcactctctc base pairs
cacacataaatcataaaacagtaagatacgtacacacctaaaagaccttacaccttttgtatgtcgtgagagag 2926 to 3000

                                        MseI   HaeIII                HaeIII
tacaccacacacacttctagtatatgtgtacacgttaatgggccaacactagacacatggcccaacatccccct base pairs
atgtggtgtgtgtgaagatcatatacacatgtgcaattacccggttgtgatctgtgtaccgggttgtaggggga 3001 to 3075

                     EcoRV
caagatgggcgatagatatcaatcatccccatcttgctacataacacatcacactcttttactcctatacccta base pairs
```

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FIG. 5

Continued

gttctacccgctatctatagttagtaggggtagaacgatgtattgtgtagtgtgagaaaatgaggatatgggaat 3076 to 3150

HinfI

gtcaagcaatctgctatttgaccttttgagtttacatgattcaactctaaagtaccattatctaacttctctttg base pairs
cagttcgttagacgataaactggaaaactcaaatgtactaagttgagatttcatggtaatagattgaagagaaac 3151 to 3225

ClaI
HinfI

atgaagaatcgatcaatttccacatgttttgttctatcatgttgaactggattgttagctatattcatggctgac base pairs
tacttcttagctagttaaaggtgtacaaaacaagatagtacaacttgacctaacaatcgatataagtaccgactg 3226 to 3300

TaqI
Sau3AI

HinfI MseI 18eF

ttattatcacaccataacttcagggagtcttttcttaatacattcaactctgataagagacccttatccatagc base pairs
aataatagtgtggtattgaagtccctcagaaaagaattatgtaagttgagactattctctgggaataggtatcg 3301 to 3375

HaeIII

atctcacatatcgcaagggccatagctcggtattctgcttcggcggtggaacgggataccacagattgtttcttg base pairs
tagagtgtatagcgttcccggtatcgagccataagacgaagccgccaccttgccctatggtgtctaacaaagaac 3376 to 3450

cttctccatgatactaaatttcctccaacaaacacacaatatcctgaagttgaccttctatcatcaaggcaacta base pairs
gaagaggtactatgatttaaaggaggttgtttgtgtgttataggacttcaactggaagatagtagttccgttgat 3451 to 3525

ScrFI
MseI EcoRII

ccccagtctgcatcagagtaaccttccacctttagatgaccatgacctttaaagattattccctttccaggacaa base pairs
ggggtcagacgtagtctcattggaaggtggaaatctactggtactggaaatttctaataagggaaaggtcctgtt 3526 to 3600

BstNI

TaqI

gtcttcaagtatcgcagtatacgatacactgcatcaagatgtccacttctggggtcatgcatatatcgactcacc base pairs
cagaagttcatagcgtcatatgctatgtgacgtagttctacaggtgaagaccccagtacgtatatagctgagtgg 3601 to 3675

HinfI

EcoRV

acactgactgcatatgtgatatcaggtcttgtatggcacaagtagatgagccgtccaacaagtctttgatacctt base pairs
tgtgactgacgtatacactatagtccagaacataccgtgttcatctactcggcaggttgttcagaaactatggaa 3676 to 3750

Sau3AI HinfI HinfI TaqI HaeIII

tctttattcacaggatcaccagattcagcacataatttatgattcaagtcgataggtgttgctacaggccgacac base pairs
agaaataagtgtcctagtggtctaagtcgtgtattaaatactaagttcagctatccacaacgatgtccggctgtg 3751 to 3825

Sau3AI Sau3AI

cccaacatacctgtttcatcaagtagatctaaaacatatttcctttgggagagaactattccttttggagatcga base pairs
gggttgtatggacaaagtagttcatctagattttgtataaaggaaaccctctcttgataaggaaaacctctagct 3826 to 3900

BglII TaqI

Sau3AI HinfI

gcaatctcaataccaagaaagtatttgagatgaccaagatctttaacctcaaattccttacttagattcttcttt base pairs
cgttagagttatggttctttcataaactctactggttctagaaattggagtttaaggaatgaatctaagaagaaa 3901 to 3975

BglII MseI

Sau3AI

agacatgcaatctcaagatcggcatcacctgtaataataatatcatccacatacacagctagaattgcaattcgt base pairs
tctgtacgttagagttctagccgtagtggacattattattatagtaggtgtatgtgtcgatcttaacgttaagca 3976 to 4050

Sau3AI

cgtccaaagtgttgataaaaaacagtgtgatctccgttgcattgtttatatcccatgctacatattgcacgtcta base pairs
gcaggtttcacaactattttttgtcacactagaggcaacgtaacaaatatagggtacgatgtataacgtgcagat 4051 to 4125

TaqI

aatctgtcaaaccatgctcttggggactgcttgagaccatacaatgatttttcaatcgacaaactttcccaatt base pairs

FIG. 5

Continued

```
ttagacagtttggtacgagaacccctgacgaactctggtatgttactaaaaagttagctgtttgaaagggttaa 4126 to 4200

                    ScrFI
                  EcoRII  Sau3AI
gtctcaggctttgacaatccaggagggatctccatatagacctcctcttgcaaatcaccatgtaagaaagcattc base pairs
cagagtccgaaactgttaggtcctccctagaggtatatctggaggagaacgtttagtggtacattctttcgtaag 4201 to 4275
                    BstNI

 MseI                       HaeIII                      Sau3AI
ttaacatctagttgatacaagggccatccaaaatttgcagcacaagagatcaatgtccttacagtactcatttt base pairs
aattgtagatcaactatgttcccggtaggttttaaacgtcgtgttctctagttacaggaatgtcatgagtaaaa 4276 to 4350

gccactggtgcaaatgtctcatcataatcaattccatatgtttgactataccctcttgcaaccaatcttgcttta base pairs
cggtgaccacgtttacagagtagtattagttaaggtatacaaactgatatgggagaacgttggttagaacgaaat 4351 to 4425

tatcgttctacccttccttctgggttttgcttcacagtgaatacccatttacaactaactgccttctttccttta base pairs
atagcaagatgggaaggaagacccaaaacgaagtgtcacttatgggtaaatgttgattgacggaagaaaggaaat 4426 to 4500
                                                          18iR
                                     XbaI      MseI
ggtagtttctcaaattcccaagtttgatttttttctagagctttaagctcctccaacattgcctcacgccagtta base pairs
ccatcaaagagtttaagggttcaaactaaaaaaagatctcgaaattcgaggaggttgtaacggagtgcggtcaat 4501 to 4575

gaattacattgtgcttctttccaatctcttggaattgctacggaatgcaatgatgcaacaaatgctctatatgat base pairs
cttaatgtaacacgaagaaaggttagagaaccttaacgatgccttacgttactacgttgtttacgagatatacta 4576 to 4650

                                                                      HinfI
ggtgacaaagacgcatatgagacataattgctaatgtcatgttcatatccataccttgttgggggactccagct base pairs
ccactgtttctgcgtatactctgtattaacgattacagtacaagtataggtatggaacaaccccctgaggtcga 4651 to 4725

          HhaI
ttagcacgcgctccttttcgtattgcaatgggcaaatcataagtgtcataatcttcagtttctccatgagacgtc base pairs
aatcgtgcgcgaggaaaagcataacgttacccgtttagtattcacagtattagaagtcaaagaggtactctgcag 4726 to 4800

aaaggtacatttatagcctctaatgtgtttggagagaactgctcagtacttgatgctgaattggtttcaggagcc base pairs
tttccatgtaaatatcggagattacacaaacctctcttgacgagtcatgaactacgacttaaccaaagtcctcgg 4801 to 4875

tgaggttgcacatgggactttcttcttgtatatacttcgcccttatatcgtaagtcgtctccacaagatttatta base pairs
actccaacgtgtaccctgaaagaagaacatatatgaagcgggaatatagcattcagcagaggtgttctaaataat 4876 to 4950

ttctcgtgactaggatgtgtctccaattcacttggcattacttgcatcttttgagaagcaccaatcaccacttcc base pairs
aagagcactgatcctacacagaggttaagtgaaccgtaatgaacgtagaaaactcttcgtggttagtggtgaagg 4951 to 5025

                         HinfI                      TaqI
attttatttggttgttgttccattgaatcaaccattctgttctccccctctcgactagcttcatctgtgctagta base pairs
taaaataaaccaacaacaaggtaacttagttggtaagacaagagggggagagctgatcgaagtagacacgatcat 5026 to 5100
                           16iR

      HinfI              Sau3AI
gagacagaatcaagaaaaaaatttagatctgtcttctcaccatagaaaggcacagtctctctaaatgtaacatcc base pairs
ctctgtcttagttctttttttaaatctagacagaagagtggtatctttccgtgtcagagagatttacattgtagg 5101 to 5175
                         BglII

                                 HinfI                           PstI
atgcttacaaacaaacgtcgttcactaggactccaacacttgtatccctttttgccctgcaggatatccaacaaaa base pairs
tacgaatgtttgtttgcagcaagtgatcctgaggttgtgaacatagggaaaacgggacgtcctataggttgtttt 5176 to 5250
                                                                   EcoRV

                   BamHI                       Sau3AI
atgcacttcacagcacgaggatccaacttccccacctgaggtctatgatctctgacaaaacatgtacatccaaaa base pairs
tacgtgaagtgtcgtgctcctaggttgaaggggtggactccagatactagagactgttttgtacatgtaggtttt 5251 to 5325
```

FIG. 5

Continued

```
                          Sau3AI
                                                     HinfI          HinfI
atttaggtggaaccacaaacttattctcaccgagaagaatctcacatggagtcttcattgcaagtatttttgaa base pairs
taaaatccaccttggtgtttgaataagagtggctcttcttagagtgtacctcagaagtaacgttcataaaaactt 5326 to 5400

        MseI
ggagtgcgattaataagatatgtggcagtcaatacagcttcactccataggaacttcggaacattcattgtaaac base pairs
cctcacgctaattattctatacaccgtcagttatgtcgaagtgaggtatccttgaagccttgtaagtaacatttg 5401 to 5475
           14iR
                                            HinfI
atcagcgaacgagcaacttccaaaatgtgacgattcttcctttcagccacaccatttgtggaggtgtatcagga base pairs
tagtcgcttgctcgttgaaggttttacactgctaagaaggaaagtcggtgtggtaaaacacctccacatagtcct 5476 to 5550

                                                     MseI
caggatgtctgatgtaatataccatttcttgacagaaatgcattaaatccccttgtttacatactcggttccattg base pairs
gtcctacagactacattatatggtaaagaactgtctttacgtaatttagggaacaaatgtatgagccaaggtaac 5551 to 5625

                              11iF      HinfI
tctggtcttaggatttgacttgagtattgaattgattctcaactagtgcacaaaaattttgaaaacacttcaat base pairs
agaccagaatcctaaaactgaactcataacttaactaagagttgatcacgtgtttttaaaacttttgtgaagtta 5626 to 5700

                        12iF                             TaqI
acttcatctttatgcttcatcacatagacccaagtcattccgagaaaaacaatcgataaagtaacaaagtacttc base pairs
tgaagtagaaatacgaagtagtgtatctgggttcagtaaggctctttttgttagctatttcattgtttcatgaag 5701 to 5775
                                                         ClaI

     MseI
atcccattaatagaagtcacaggacatgtccaaacatcagaatgaactagcacaaaaggagatatactcctgata base pairs
tagggtaattatcttcagtgtcctgtacaggtttgtagtcttacttgatcgtgttttcctctatatgaggactat 5776 to 5850

  TaqI
cctcgactaatataagatgtccttgtgtgttttgcaaactcacaggcatcacacaatagcttgctttatccacc base pairs
ggagctgattatattctacaggaacacacaaaacgtttgagtgtccgtagtgtgttatcgaacgaaaataggtgg 5851 to 5925

                  HindIII
ccactcattacatcaggaaaagctttgcatatcttatcaaaagaaagatgccctaatctacaatgcaagagcatc base pairs
ggtgagtaatgtagtccttttcgaaacgtatagaatagttttctttctacgggattagatgttacgttctcgtag 5926 to 6000

                                                                      Sau3AI
actgcaacctccttctcttccattcttgttgccagcatagtgcatattgtaccattagtcccctcatgatccata base pairs
tgacgttggaggaagagaaggtaagaacaacggtcgtatcacgtataacatggtaatcaggggagtactaggtat 6001 to 6075

                 ScrFI
               EcoRII                                            MseI
taccacaatccattacgcctggtagctgtcccaagtctcttccctgtttccctctcctgaattaaacaattatct base pairs
atggtgttaggtaatgcggaccatcgacagggttcagagaagggacaaagggagaggacttaatttgttaataga 6076 to 6150
                 BstNI

TaqI                   Sau3AI                EcoRV
cgatcaagaataatacgacaatccaattgatcaaccaaggcacttagtgatatcaagttgacaggaaaggttggc base pairs
gctagttcttattatgctgttaggttaactagttggttccgtgaatcactatagttcaactgtcctttccaaccg 6151 to 6225
 Sau3AI

                  MseI
acatacaaaactgatgacaacttaatagatggagtgcattgcactgtgccaacacccttgatgggttgtggtgta base pairs
tgtatgttttgactactgttgaattatctacctcacgtaacgtgacacggttgtgggaactacccaacaccacat 6226 to 6300

                                             EcoRV
ccatcagcagtttgtataatttctttacgtgtggggggatatcttatatatgatgtaaattcactggacgtgcct base pairs
ggtagtcgtcaaacatattaaagaaatgcacacccccctatagaatatatactacatttaagtgacctgcacgga 6301 to 6375
```

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FIG. 5

Continued

```
                    HinfI              MseI
gtgacatgctttgatgctcctgagtctaaaatccattttaactgtgtgacctgtgtgggtacaaaagcatgagca base pairs
cactgtacgaaactacgaggactcagattttaggtaaaattgacacactggacacacccatgttttcgtactcgt 6376 to 6450

                                                HinfI Sau3AI
taattaccttcatcagtgtaggcgaagtggacaaaatcccctgtgtgagactcctgatctttatctccagagatt base pairs
attaatggaagtagtcacatccgcttcacctgttttaggggacacactctgaggactagaaatagaggtctctaa 6451 to 6525

tgatttttcttcctcaactttgtttcatcttcgtgttccataaatgtttcaagttcttcttgtgtagttgctgca base pairs
actaaaaagaaggagttgaaacaaagtagaagcacaaggtatttacaaagttcaagaagaacacatcaacgacgt 6526 to 6600

                                                                 MseI
ttcgcccttgcccaactcctgcctccacgacctctgccgcctctaggagcccctcttcctctcccacgattaact base pairs
aagcgggaacgggttgaggacggaggtgctggagacggcggagatcctcggggagaaggagagggtgctaattga 6601 to 6675

ttggaaggcttagaacaattacgtgcaatatgtccaacattaccacaattgtaacattctctagtatctttggtt base pairs
aaccttccgaatcttgttaatgcacgttatacaggttgtaatggtgttaacattgtaagagatcatagaaaccaa 6676 to 6750

                                                                   ScrFI
                                                      HinfI  HinfI EcoRII
ctcatagctgaaaacacaggatgaggcggcgtttgagaactttctctcatcactttgagtcttgactcctcctgg base pairs
gagtatcgacttttgtgtcctactccgccgcaaactcttgaaagagagtagtgaaactcagaactgaggaggacc 6751 to 6825
                                                                   BstNI

                                                                 TaqI
gatatggcagctatggcttcttgtaggctaggaagagtggattgatgaaacatggaggcacgtcttccctcgaac base pairs
ctataccgtcgataccgaagaacatccgatccttctcacctaactactttgtacctccgtgcagaagggagcttg 6826 to 6900
                                                   13iR

tctgagtttagcccccttagcaattgaagtacacgtctttttccacccatttcttcgcccaagcaacacactct base pairs
agactcaaatcgggggaatcgttaacttcatgtgcagaaaaaggtgggtaaagaagcgggttcgttgtgtgaga 6901 to 6975

                  Sau3AI   Sau3AI
gagtgtggtagctcaataggatcataatgatcaacatcagcccataaacattgtaactcctgaacgtactccgcc base pairs
ctcacaccatcgagttatcctagtattactagttgtagtcgggtatttgtaacattgaggacttgcatgaggcgg 6976 to 7050

  Sau3AI                               Sau3AI     13iF
acagatcgctccccctgtttgatattatggaggcgatcttcagtctccaccatcaacataacatttccagctccc base pairs
tgtctagcgagggggacaaactataatacctccgctagaagtcagaggtggtagttgtattgtaaaggtcgaggg 7051 to 7125

                                        PstI                            HinfI
gagtacatttcttcaagtgctttccacatttctgcagcacttatgattgtatcaacagtgctagcaattgctgga base pairs
ctcatgtaaagaagttcacgaaaggtgtaaagacgtcgtgaatactaacatagttgtcacgatcgttaacgacct 7126 to 7200

                                                                MseI
atcatagaactcaacatccacgctgccactaaagagtttatagcatcccagtctttccattcatcacttaactta base pairs
tagtatcttgagttgtaggtgcgacggtgatttctcaaatatcgtagggtcagaaaggtaagtagtgaattgaat 7201 to 7275

                                          HinfI
                         MseI        XhoI                              XbaI
tccttgggctcaacgacatctcctttaacatagccctcgagtctctttgccttcaataatcgcaatgctcttcta base pairs
aggaacccgagttgctgtagaggaaattgtatcgggagctcagagaaacggaagttattagcgttacgagaagat 7276 to 7350
                                       TaqI                           S4iR

                                      MseI
gaccatgccaaataatttttcaccccttctaacttaatctcatttggcattaggtctatcttctgaactggttct base pairs
ctggtacggtttattaaaaagtggggaagattgaattagagtaaaccgtaatccagatagaagacttgaccaaga 7351 to 7425
                                               12iR
                   MseI                                      TaqI
atatgagcaacattgtctttaattgatgatggagcctcatcccttttttgctgacagtaattcgaccaatttacca base pairs
tatactcgttgtaacagaaattaactactacctcggagtagggaaaaaacgactgtcattaagctggttaaatggt 7426 to 7500
```

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FIG. 5

Continued

```
                                                                       PvuII
agaacctgctcaattccttgatttcccccataatatatatgtaataataactaggaaactacttgggcagctgc base pairs
tcttggacgagttaaggaactaaagggggtattatatatacattattattgatcctttgatgaacccgtcgacg 7501 to 7575

                                          EcoRII
                                       ScrFI
    Sau3AI            XbaI       EcoRII  BstNI   HhaI                             Sau3AI
gtcaagatctgggtcacaacgtctagaagccaggaccaggagcgcctcctcttcctcctcctccgagctggatgg base pairs
cagttctagacccagtgttgcagatcttcggtcctggtcctcgcggaggagaaggaggaggaggctcgacctacc 7576 to 7650
    BglII                              BstNI ScrFI
                                        AvaII

                                                             HpaII
atctcagtcacaggacgcgggcagcagggggggagcagcagcacctgtgtgccggcagcttcctcaagggttggac base pairs
tagagtcagtgtcctgcgcccgtcgtcccccctcgtcgtcgtggacacacggccgtcgaaggagttcccaacctg 7651 to 7725
                                                             MspI

          PvuII                         Sau3AI
gagctgcggcagctggagagcctcccaagcacccctatctccagatccttgtcgccgacgagtgcccgcgtccac base pairs
ctcgacgccgtcgacctctcggagggttcgtggggatagaggtctaggaacagcggctgctcacgggcgcaggtg 7726 to 7800
                                      S6RN

                                                                       ScrFI
       HaeIII                                                Eco0109I  BstNI
gtccttggccgccctcgccttgtcggcggtggcgtcctctgtgctgtggctcgggacctgtccctggcctcctgc base pairs
caggaaccggcgggagcggaacagccgccaccgcaggagacacgacaccgagccctggacagggaccggaggacg 7801 to 7875
                                                             AvaII   EcoRII
                                                                        HaeIII

                                        ScrFI
HhaI                                  EcoRII                      TaqI
gcggcctccctgctggcgtcggtgtactcgcccgtcttctgcctggtcacgtccctcgcctccctcgatcgctcg base pairs
cgccggagggacgaccgcagccacatgagcgggcagaagacggaccagtgcagggagcggagggagctagcgagc 7876 to 7950
   HaeIII                               BstNI                        Sau3AI

               HaeIII     HaeIII   Sau3AI                      TaqI HaeIII
tgtgcctcggcggcctccttcggccgtcgctgatctccttctcggtggtcttctccgtcgaggccgaagacactc base pairs
acacggagccgccggaggaagccggcagcgactagaggaagagccaccagaagaggcagctccggcttctgtgag 7951 to 8025

                             ScrFI
                           EcoRII
gtcaccgcgacgccatcgccgttgagcctggctctgataccatgtggatttttctggaatgtggaaaacatacag base pairs
cagtggcgctgcggtagcggcaactcggaccgagactatggtacacctaaaaagaccttacaccttttgtatgtc 8026 to 8100
                  11iR       BstNI

                                        MseI    HaeIII          HaeIII
cactctctctacaccacacacacttctagtatatgtgtacacgttaatgggccaacactagacacatggcccaac base pairs
gtgagagagatgtggtgtgtgtgaagatcatatacacatgtgcaattacccggttgtgatctgtgtaccgggttg 8101 to 8175

7F
agcatgtcaagtggcatagcactcacatttgtcatggcaggttatcaattctttggtgtccataagtgtggatgg base pairs
tcgtacagttcaccgtatcgtgagtgtaaacagtaccgtccaatagttaagaaaccacaggtattcacacctacc 8176 to 8250

HaeIII                                8eF
ccacaagcatgaccctagacgtgtttttctttctgaggaaaagaacgacaatgtgcttgattgcattatctccaa base pairs
ggtgttcgtactgggatctgcacaaaaagaaagactccttttcttgctgttacacgaactaacgtaatagaggtt 8251 to 8325
                                          8eR

            Sau3AI                Pst I
ggtcaagatagtccatgttgatccaaatgtaagtttgctgcagtttgctgagagctttgtggttttgctatacac base pairs
ccagttctatcaggtacaactaggtttacattcaaacgacgtcaaacgactctcgaaacaccaaaacgatatgtg 8326 to 8400
                                                              5RN
```

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FIG. 5

Continued

```
                                                  BamHI                PvuII
ataatgtttctgactaccattgttttgttgcctacttgccttagatggatccaaaagccaaggctcagctgatag base pairs
tattacaaagactgatggtaacaaaacaacggatgaacggaatctacctaggttttcggttccgagtcgactatc 8401 to 8475
                                                  Sau3AI

agagttgcgacctatactatgacatgtcttactctgttgcatattctacatttgctaatatctcgtctggtaatt base pairs
tctcaacgctggatatgatactgtacagaatgagacaacgtataagatgtaaacgattatagagcagaccattaa 8476 to 8550

                                                                   MseI
ccttctgcatcatctttttggttgactagctgaatgcagttagctttgccaaagagttaaatacatgagttgtt base pairs
ggaagacgtagtagaaaaaccaactgatcgacttacgtcaatcgaaacggtttctcaatttatgtactcaacaa 8551 to 8625

       TaqI                                                          MseI     MseI
cctgcactcgaaaagggatgtcaataatgtccacaaactctgaaaatgtattttagatacttaacttgttaagt base pairs
ggacgtgagcttttccctacagttattacaggtgtttgagacttttacataaaatctatgaattgaacaattca 8626 to 8700

cagtaaaacctgtcagatacttgggttttgggtacgattaccatccttatgtgagtaaaactcgtcaagggatgt base pairs
gtcattttggacagtctatgaacccaaaacccatgctaatggtaggaatacactcattttgagcagttccctaca 8701 to 8775

                                         TaqI        Seq2FN
caatgacgtgttgattgtgtattagatattctgtttgttcgaaagctaatctacacaaacagcttatgtaatgta base pairs
gttactgcacaactaacacataatctataagacaaacaagctttcgattagatgtgtttgtcgaatacattacat 8776 to 8850

                              HindIII                                HaeIII
aaacctcaaacaaacttgcctcttcataagcttaggtttataggattagcgtttagtgcatgtaaggcctatttg base pairs
tttggagtttgtttgaacggagaagtattcgaatccaaatatcctaatcgcaaatcacgtacattccggataaac 8851 to 8925

                         BstNI            ScrFI                          ScrFI
       HaeIII          SacI             EcoRII                 TaqI  EcoRII
cttcacggcctccctgccgagctcctggctagacagccatcctggccgtaggtgcccgaaatcgaacacctggga base pairs
gaagtgccggagggacggctcgaggaccgatctgtcggtaggaccggcatccacgggctttagcttgtggaccct 8926 to 9000
                       EcoRII           BstNI                            BstNI
                         ScrFI             HaeIII

                          ScrFI
                        EcoRII
gccacgtttgcactagcaggttttcctgggtgcaaaccaaacacgcctatagtgttcaagtataactgaattggt base pairs
cggtgcaaacgtgatcgtccaaaaggacccacgtttggtttgtgcggatatcacaagttcatattgacttaacca 9001 to 9075
                          BstNI

                     MseI
gctcacctttgtctaatagcttaagtttttggttttcatcggtgcatgcaactccatactcaatagtcaatatga base pairs
cgagtggaaacagattatcgaattcaaaaaccaaaagtagccacgtacgttgaggtatgagttatcagttatact 9076 to 9150

                                     BstNI
                       XhoI     HinfI
tatagtgttcaagcatagaactctcgagtttgaatcctggcaggggcaatcaaataaaataattgcagcttaccc base pairs
atatcacaagttcgtatcttgagagctcaaacttaggaccgtccccgttagtttattttattaacgtcgaatggg 9151 to 9225
                        TaqI        EcoRII
                                     ScrFI

                      S3iF
ctatttctacgtttgagcacatgagggagagtgttgaattataagtgtgttctccatctttctctaacagatgaa base pairs
gataaagatgcaaactcgtgtactccctctcacaacttaatattcacacaagaggtagaaagagattgtctactt 9226 to 9300

                              HinfI          MseI                MseI
ctggtttgtgcatgtaactcaatatgatatttgagtcaaatgtttactttaaaatcatagttgatgcaatttaat base pairs
gaccaaacacgtacattgagttatactataaactcagtttacaaatgaaattttagtatcaactacgttaaatta 9301 to 9375
```

FIG. 5

Continued

aacatatttttttggtctcgtgtgagggagtgtacgtataactgaattgcacacatttccttatagcttaggttt base pairs
ttgtataaaaaaaccagagcacactccctcacatgcatattgacttaacgtgtgtaaaggaatatcgaatccaaa 9376 to 9450

Sau3AI
ttgactgcaactgttggtgcatgtagctcaataactaaagttgatctggacagtctacagtgaataagtttgaca base pairs
aactgacgttgacaaccacgtacatcgagttattgatttcaactagacctgtcagatgtcacttattcaaactgt 9451 to 9525

cttgtaaaatgtgcatgtatttttacaaacgctggcactttttcctaatagaaaatgggcagtcaggcagtgat base pairs
gaacattttacacgtacataaaaatgtttgcgaccgtgaaaaaggattatcttttacccgtcagtccgtcacta 9526 to 9600

Sau3AI
accgcttcgggtatttcttctgatgatgtggatctggagacgtcatctagtatgccaacgaggacagcaaccctt base pairs
tggcgaagcccataaagaagactactacacctagacctctgcagtagatcatacggttgctcctgtcgttgggaa 9601 to 9675

Sau3AI
cttgatctgtattctggctgtgggggcatgtctactggtctttgcttgggtgcagctctttctggcttgaaactt base pairs
gaactagacataagaccgacacccccgtacagatgaccagaaacgaacccacgtcgagaaagaccgaactttgaa 9676 to 9750

Sau3AI
gaaactgtaatcttctaactagtcatctgttggatagaatatgttcacgatctcagaacttattctattgttctg base pairs
ctttgacattagaagattgatcagtagacaacctatcttatacaagtgctagagtcttgaataagataacaagac 9751 to 9825

MseI
gcttgcagcgatgggctgttgatttcaacagttttgcgtgccaaagtttaaaatataatcatccacagactgagg base pairs
cgaacgtcgctacccgacaactaaagttgtcaaaacgcacggtttcaaattttatattagtaggtgtctgactcc 9826 to 9900

HinfI
tatggatagtaaacttcatcttggattccatctgttctgtcagctactcttacaaagtgtctggattttggatg base pairs
atacctatcatttgaagtagaacctaaggtagacaagacagtcgatgagaatgtttcacagacctaaaacctac 9901 to 9975

MseI
taggtgcgaaatgagaaagccgatgagtttcttgccctccttaaggaatgggcagttctatgcaaaaaatatgtc base pairs
atccacgctttactctttcggctactcaaagaacgggaggaattccttacccgtcaagatacgttttttatacag 9976 to 10050

HinfI Sau3AI
caagatgtggattcaaatttagcaagctcagaggatcaagcggatgaagacagccctcttgacaaggacgaattt base pairs
gttctacacctaagtttaaatcgttcgagtctcctagttcgcctacttctgtcgggagaactgttcctgcttaaa 10051 to 10125
Seq2RN

HindIII MseI
gttgtagagaagcttgtcgggatatgttatggtggcagtgacagggaaaatggcatctatttaaggtacttcag base pairs
caacatctcttcgaacagccctatacaataccaccgtcactgtccctttaccgtagataaattccatgaagtc 10126 to 10200

HinfI MseI
tgtcatttgttcatttctacttgattccaacaaaaaatcaattacttaagcctgtcaaacgatggatatttctg base pairs
acagtaaacaagtaaagatgaactaaggttgttttttagttaatgaattcggacagtttgctacctataaagac 10201 to 10275

PstI HaeIII
tatatttgctgtaacgctagatttctgcaggtccagtgggaaggatacggccctgaggaggatacatgggaacc base pairs
atataaaacgacattgcgatctaaagacgtccaggtcacccttcctatgccgggactcctcctatgtacccttgg 10276 to 10350
AvaII

gattgataacttgaggttagtgtatggtatatcgtctgcttgttgccttgtatacctatttgcatctaatccttg base pairs
ctaactattgaactccaatcacataccatatagcagacgaacaacggaacatatggataaacgtagattaggaac 10351 to 10425
Seq1RN

HinfI

FIG. 5
Continued

ttttgcaaaccagtgactgcccgcagaaaattagagaatttgtacaagaagggcacaaaagaaagattctcccac base pairs
aaaacgtttggtcactgacgggcgtcttttaatctcttaaacatgttcttcccgtgttttctttctaagagggtg 10426 to 10500
S3eR

EcoRV
tgcctgtgagtatttagttcgttgtgatttgctcgctatttgtttagctcccctttttttatttggtgatatctg base pairs
acggacactcataaatcaagcaacactaaaacgagcgataaacaaatcgaggggaaaaaataaaccactatagac 10501 to 10575

HaeIII MseI
cctattttattctttcaaagggtgatgttgatgtcatttgtggaggcccaccatgccaaggtatcagtgggttta base pairs
ggataaaataagaaagtttcccactacaactacagtaaacacctccgggtggtacggttccatagtcacccaaat 10576 to 10650

3F
atcggtacagaaaccgtgatgagccactcaaagatgagaaaaacaaacaaatggtgactttcatggatattgtgg base pairs
tagccatgtctttggcactactcggtgagtttctactctttttgtttgtttaccactgaaagtacctataacacc 10651 to 10725
3R

cgtacttgaagcccaagtatgttctcatggaaaatgtggtggacatactcaaatttgcggatggttacctaggaa base pairs
gcatgaacttcgggttcatacaagagtaccttttacaccacctgtatgagtttaaacgcctaccaatggatcctt 10726 to 10800

HhaI 1F
aatatgctttgagctgccttgttgctatgaagtaccaagcgcggcttggaatgatggtggctggttgctatggtc base pairs
ttatacgaaactcgacggaacaacgatacttcatggttcgcgccgaaccttactaccaccgaccaacgataccag 10801 to 10875

Race2A
tgccacagttcaggatgcgtgtgtacctctggggtgctctttctt~~ccatg~~gtctgttctgtaccttgctgtttta base pairs
acggtgtcaagtcctacgcacacatggagaccccacgagaaagaa~~ggtac~~cagacaagacatggaacgacaaaat 10876 to 10950
Race2B

ScrFI
HinfI EcoRII
tatgcttcgctagattcatattgcactgttggctgctggctaaccaggtgtacgtgtatttgacaatttag~~gtgc~~ base pairs
atacgaagcgatctaagtataacgtgacaaccgacgaccgattggtccacatgcacataaactgttaaatc~~cacg~~ 10951 to 11025
BstNI

Race1A 4F
~~tccctaagtatcc~~tctgcccaactatgatgttgtagtacgtggaggagcccctaatgcctttcggtgagtgcaat base pairs
~~agggattcatagg~~agacgggttgatactacaacatcatgcacctcctcggggattacggaaagccactcacgtta 11026 to 11100
Race1B RaceRT

cacaaaccactactatgaaatcatgtggaatgtgtaaaatacgctgaccaactgaatttgttgcagcaatgtatg base pairs
gtgtttggtgatgatactttagtacaccttacacattttatgcgactggttgacttaaacaacgtcgttacatac 11101 to 11175

gttgcatatgacgagacacaaaaaccatccctgaaaaaagccttgcttcttggcgatgcaatttcagatttacca base pairs
caacgtatactgctctgtgtttttggtagggacttttttcggaacgaagaaccgctacgttaaagtctaaatggt 11176 to 11250

MseI PstI
aaggcaagtgttctgtcaagttcatgcatttctcagtgagcatgctatttaactcttctctgcaggttcaaaatc base pairs
ttccgttcacaagacagttcaagtacgtaaagagtcactcgtacgataaattgagaagagacgtccaagttttag 11251 to 11325

EcoRI HhaI TaqI
accagcctaacgatgtgatggagtatggtggttccccaagaccgaattccagcgctacattcgactcagtcgtaa base pairs
tggtcggattgctacactacctcataccaccaaggggttctggcttaaggtcgcgatgtaagctgagtcagcatt 11326 to 11400
HinfI

HaeIII MseI
aggtaaaaaccccgtgaactactactggttggccttcactacgaatatgttaggatttaatttcagaagaaccg base pairs
tccatttttggggcacttgatgatgaccaaccggaagtgatgcttatacaatcctaaattaaagtcttcttggc 11401 to 11475

PstI AvaII
cctttttttttcttggtgcttcggtactactgcagcaagctcactcttattatcatgtcagacatgttggattggt base pairs
ggaaaaaaaagaaccacgaagccatgatgacgtcgttcgagtgagaataatagtacagtctgtacaacctaacca 11476 to 11550

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FIG. 5

Continued

```
                    AvaII                   Sau3AI              MseI
ccttcggtgaagggggctggtccagatgaaggcaagctcttggatcaccagcctttacggcttaacaacgatgat base pairs
ggaagccacttcccccgaccaggtctacttccgttcgagaacctagtggtcggaaatgccgaattgttgctacta 11551 to 11625

                   HinfI
tatgagcgggttcaacagattcctgtcaagaaggttggtggcttggtcgcatttgtgccttcctttgttgttttt base pairs
atactcgcccaagttgtctaaggacagttcttccaaccaccgaaccagcgtaaacacggaaggaaacaacaaaaa 11626 to 11700

                                                                         5F
tccccttctgaaacaatcatctctctttcctatgacagggagccaacttccgcgacctaaagggcgtgagggttg base pairs
aggggaagactttgttagtagagagaaaggatactgtccctcggttgaaggcgctggatttcccgcactcccaac 11701 to 11775

                         BamHI        TaqI
gagcaaacaatattgttgagtgggatccagaaatcgagcgtgtgaaactttcatctgggaaaccactggtatgtg base pairs
ctcgtttgttataacaactcaccctaggtctttagctcgcacactttgaaagtagaccctttggtgaccatacac 11776 to 11850
                         Sau3AI

tgctatttccgtgctgttgtttcctataactgtgcaacatttactttcccatattcaaactcataactgacgaga base pairs
acgataaaggcacgacaacaaaggatattgacacgttgtaaatgaaagggtataagtttgagtattgactgctct 11851 to 11925

                   HinfI
tgctgcaactactgtaagattcatggctaacccatgacaacatttgcacacatctttgttatctaggttcctga base pairs
acgacgttgatgacattctaagtaccgattgggtactgttgtaaaacgtgtgtagaaacaatagatccaaggact 11926 to 12000

ctatgcaatgtcattcatcaagggcaaatcactcaagtaagtttcaaaacatttttttgtttttttggggaaaa base pairs
gatacgttacagtaagtagttcccgtttagtgagttcattcaaagttttgtaaaaaaacaaaaaacccccttttt 12001 to 12075

                                         HaeIII     HhaI
gtaggttattgtttacttgtgcttacatatgatgttgcaggccgtttgggcgcctgtggtgggacaagacagttc base pairs
catccaataacaaatgaacacgaatgtatactacaacgtccggcaaacccgcggacaccaccctgttctgtcaag 12076 to 12150

                                 ScrFI
                               EcoRII            HaeIII
ctacagttgtaaccagagcagagcctcacaaccaggtcagcttcagaaaggccactcctttcgccaatccctgc base pairs
gatgtcaacattggtctcgtctcggagtgttggtccagtcgaagtctttccggtgaggaaaagcggttagggacg 12151 to 12225
                                 BstNI

                                 Sau3AI
atctgtatttactattagcgtgtgttcccatatgatcattaccgaacatgttgtccacacaggttataattcatc base pairs
tagacataaatgataatcgcacacaagggtatactagtaatggcttgtacaacaggtgtgtccaatattaagtag 12226 to 12300

ScrFI
  HinfI        Eco0109I     HpaII
cgactcaagcaagggtcctcactatccgggagaacgcaaggttacagggcttccccgattattaccgattgtttg base pairs
gctgagttcgttcccaggagtgataggccctcttgcgttccaatgtcccgaaggggctaataatggctaacaaac 12301 to 12375
               AvaII         MspI

 HaeIII                                         Sau3AI
gcccgatcaaggagaagtaagttcctgtttcaagttgcctgtaccagatctagtcactattgaaagttttcagc base pairs
cgggctagttcctcttcattcaaggacaaaagttcaacggacatggtctagatcagtgataactttcaaaagtcg 12376 to 12450
    Sau3AI                                      BglII

agcaagccattcatcagttagttacagctcttgaaagccttacctctgaacatgtgtgctttctctgatggtgat base pairs
tcgttcggtaagtagtcaatcaatgtcgagaactttcggaatggagacttgtacacacgaaagagactaccacta 12451 to 12525

                                            MspI
                                            HpaII                          EcoRII
aggtacattcaagtcgggaacgcagtggctgtccctgttgcccgggcactgggctactgtctggggcaagcctac base pairs
tccatgtaagttcagcccttgcgtcaccgacagggacaacgggcccgtgacccgatgacagaccccgttcggatg 12526 to 12600
                                            ScrFI
                                             SmaI
```

FIG. 5

Continued

```
 ScrFI
       HinfI                        PvuII
ctgggtgaatctgaggggagtgaccctctgtaccagctgcctccaagtttcacctctgttggaggacgcactgcg base pairs
gacccacttagactcccctcactgggagacatggtcgacggaggttcaaagtggagacaacctcctgcgtgacgc 12601 to 12675
 BstNI

           Eco0109I                  PstI                          Sau3AI
gggcaggcgagggcctcttcctgttggcacccctgcaggggaggtagttgagcagtaaaaggatgacagatctga base pairs
cccgtccgctcccggagaaggacaaccgtggggacgtcccctccatcaactcgtcattttcctactgtctagact 12676 to 12750
             HaeIII                                          1R     BglII
                                             TaqI
gctgagctgggcaacatccagcggcaggagcatttctggttcggttcgattcgggctcacga  base pairs
cgactcgacccgttgtaggtcgccgtcctcgtaaagaccaagccaagctaagcccgagtgct  12751 to 12812
                                               HinfI
```

FIG. 6

PROCESS	WORLD WIDE WEB SITE
sequence format conversion	http://dot.imgen.bcm.tmc.edu:9331/seq-util/Options/readseq.html
reverse complementation	http://dot.imgen.bcm.tmc.edu:9331/seq-util/Options/revcomp.html
sequence translation	http://dot.imgen.bcm.tmc.edu:9331/seq-util/Options/sixframe.html
protein information	http://www.expasy.ch/tools
sequence alignments using Clustal W	http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html
sequence comparisons using BLAST 2.0	http://www.ncbi.nlm.nih.gov/gorf/bl2.html
sequence searches using BLAST 2.0	http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=0

FIG. 7

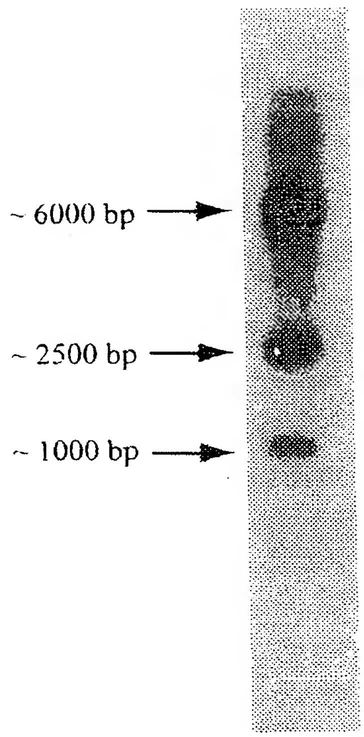


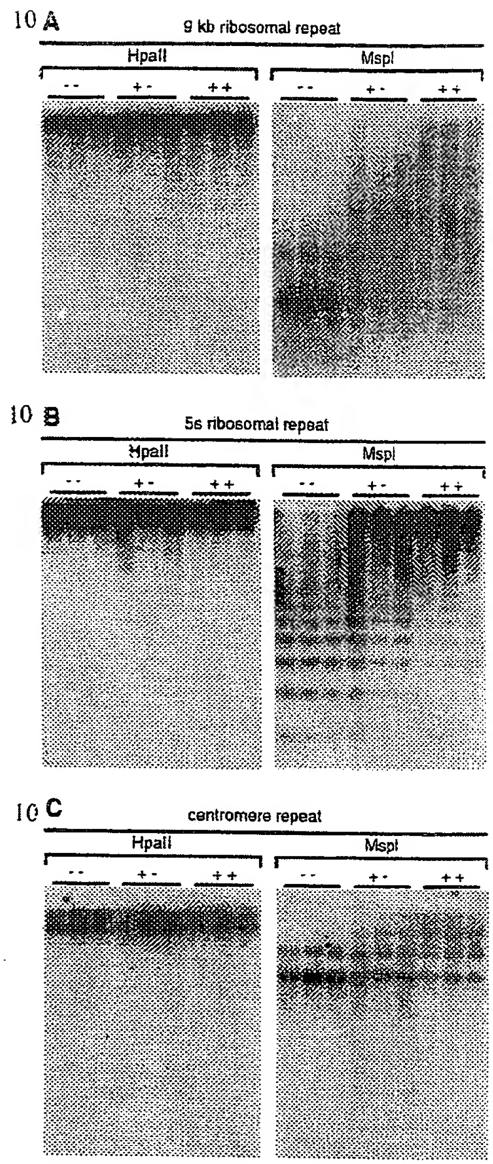
FIG. 8

```
                                                                                  NLS
zmet2      1 mapsspspaaptrvsgrkraakaeeihqnkeeeeevaaassakrsrkaassgkkpksppkqakpgrkkkgdaemkepveddvcaeepdeeelaMGeeeaeeqamQeEvvavaAGsPg
CMT1       1 -----------------------------------------------------------------------------------------------MAarnkqkkraEpEsdlcfAGkP-

         NLS
zmet2    118 kkrvgrrnAaaaAgdHePefigSpvaadEArSnwPkryGrstaAKKpDEEeelkARCHYrsAkVDnVVycLgDDVYVkAgeneadYIGRItEfFEGtDqchYftcRWFFRaEDTVIn
CMT1      23 -------mSvveStiRwPhryqSkktklQApTkkPankG----GKKeDEEiikqAKCHFdkAlVDgVLinLnDDVYVtGlpgklkFIAKViElFEAdDgvpYcrfRWYYRpEDTLIe

                                                                                                                 Motif I
zmet2    235 slvsisvdgHkhdPRRVFLSeEkNDNvLdCIiSKVkIvHVd-PnMdpKakaqLIesCDlYYDMsYsVaYstFaniSsENgqsGSDtASgISSDdvdLetsssMptrtatLLDLYSGC
CMT1     130 rfs------HlvqPKRVFLSnDeNDNpLtCIwSKVnIaKVplPkItsRieqrVIppCDyYYDMkYeVpYlnFt--SaDD---GSDaSSsLSSD-saLncfenLhkdekfLLDLYSGC
zmet1   1084                                                                                                                 LDIFAGC
MET1    1096                                                                                                                 LDIFAGC

     Motif I
zmet2    351 GGMSTGLCLGAALSGLKLeTRWAVDFNsFACQSLKyNHPQTEVRNEkADEFLALLKEWavLCkKY-----------VqDVDsnLasseDQaDEdSp---LDkdEFVVEKlVGIcYGg
CMT1     228 GAMSTGfCMGASISGVKLiTKWSVDiNkFACdSLKlNHPETEVRNEaAEDFLALLKEWkrLCeKFslvsstepvesIsELEdeeveenDdiDEaStgaeLEpgEFeVEKfLGImFGd
zmet1   1091 GGLSeGLqqAGvsf-----TKWAIEYeepAgEAfnkNHPEavV---fvDncnVILKai-------------------------MdkcgDtdDcvSt-------------------se
MET1    1103 GGLShGLkkAGvsd-----AKWAIEYeepAgQAfKqNHPEStV---fvDncnVILRai-------------------------MekggDQdDcvSt-------------------te

                   Chromodomain                                       Motif IV                                   Motif VI
zmet2    454 SD--rEnglyfkVqWeGYgpeeDTWEPiDnLsDCpQKIREFVqEGhKrKILPLPGDVDvICGGPPCQGISGFNRYRNrDePLkDEKNkQMVtFMDIVaYLKPKYVLMENVVDILKFA
CMT1     352 pqgtgEktLqlmVrWkGYnssyDTWEPysgLgNCkEKLKEYVIDGfKsHLLPLPGtVytVCGGPPCQGISGYNRYRNnEaPLeDQKNqQLLvFLDIIdFLKPnYVLMENVVDLLRFS
zmet1   1156 Aa-------------------------E----qaaKLpEvnin-----nLPVPGEVEfInGGPPCQGfSGmNRFn--qsPwskvqcemILaFLsfaeYfRPRFfLLENVrNfVsFn
MET1    1168 AN-------------------------E----laaKLtEeqks-----tLPLPGqVDfInGGPPCQGfSGmNRFn--qsswskvqcemILaFLsfadYfRPRYfLLENVrtfVsFn

                                   Motif VIII
zmet2    569 dGYLGKyALScLVAMkYQaRLGMMvAGcYGLPQfRmRVFLWGAIsSmVLPkYPLPTyDVvVRqgaPnaFsqcmVAydETQkp-SLkKALlLGDAISDLPKVqNhqpNDVMEYG-GSP
CMT1     469 kGFLARhAVAsfVAMnYQtRLGMMaAGsYGLPQlRnRVFLWAAqpSekLPpYPLPThEVakKfntPkeFkdlqVGriQmEfl-kLdnALtLADAISDLPpVtNyvaNDVMDYndAAP
zmet1   1236 kGqtfRlAVAsLLeMgYQvRfGILeAGaFGVaQsRkRaFIWAAapgeMLPdWPePmhvfaspelkitlpdgqyyAaarStaggApfRAItVrDtIgDLPKVgNgaskltLEYG-GeP
MET1    1248 kGqtfqltLAsLLeMgYQvRfGILeAGaYGVsQsRkRaFIWAAapeeVLPeWPePmhvfgVpklkislsqglhyAavrStalgApfRpItVrDtIgDLPsVeNgdsrtnkEYk-eva

zmet2    684 KTEFQRYIRLSRkDmLdwSfGegAgPdegKLlDHQPLrLNNDDyERVQqIPvKKGANFRDLkGVRVgaNNivEwDPeiervKLsSGKpLVPDYAMSFIKGKSlKPFGRLWWDEtVPT
CMT1     585 KTEFEnFIsLkRsEtLlpAcG--GdP-trRLfDHQPLvLgDDDlERVsyIPkqKGANYRDMpGVlV-hNNkaEiNPrfr-aKLkSGKnVVPaYAISFIKGKSkKPFGRLWgDEiVnT
zmet1   1352 vSwFQKkIRgS-----------------mmvLnDHiskeMNelNliRcQhIPkRpGcDWHDLpdeKVklsNgqmaDl----------ipwcLPNtAkrhnqwKg--lYGRLdWEgnfPT
MET1    1364 vSwFQKeIRgn-----------------tiaLtDHickaMNelNliRcklIPtRpGADWHDLpkrKVtlsDgrveEm----------ipfcLPNtAerhngwKg--lYGRLdWqgnfPT

                                        Mu
                     Motif IX           ▽                 Motif X
zmet2    801 VVTRAEPHN--QVIIHPtQaRVLTIRENARLQGFPDyYRLfGpIKEKYIQVGNAVAVPVArALGYcLGQAylGeSEGSDPLyqLPpsFtsvggrtagQARaspvgtpagevveq
CMT1     697 VVTRAEPHN--QcVIHPmQnRVLSVRENARLQGFPDcYKLcGtIKEKYIQVGNAVAVPVGvALGYafGmAsqGlTD-dEPVikLPfkYp-----ecmQAKdqi
zmet1   1442 sVTdpQPmgkvgMcfHPdQdRIITVRECARsQGFPDsYefaGnIqnKhrQIGNAVppPLAyALGrkLkEAvdkrqEASagV-paP
MET1    1454 sVTdpQPmgkvgMcfHPeQhRILTVRECARsQGFPDsYefaGnInhKhrQIGNAVppPLAfALGrkLkEAlhlkks---P-qhqP
```

FIG. 9

	SAM binding		Cytosine binding	
Motif	M.*HhaI*	*zmet2a*	M.*HhaI*	*zmet2a*
I	Phe18	Try347		
II	Glu40	Gln407		
	Trp41	Trp408		
III	Asp60	Asp428		
IV	Pro80	Pro516	Phe79	Pro515
	Gln82	Gln82	Cys81	Cys517
V	Leu100	Val542		
VI			Glu119	Glu559
			Asn120	Asn560
			Val121	Val561
VIII			Arg165	Arg605
X	Asn304	Asn851		

FIG. 10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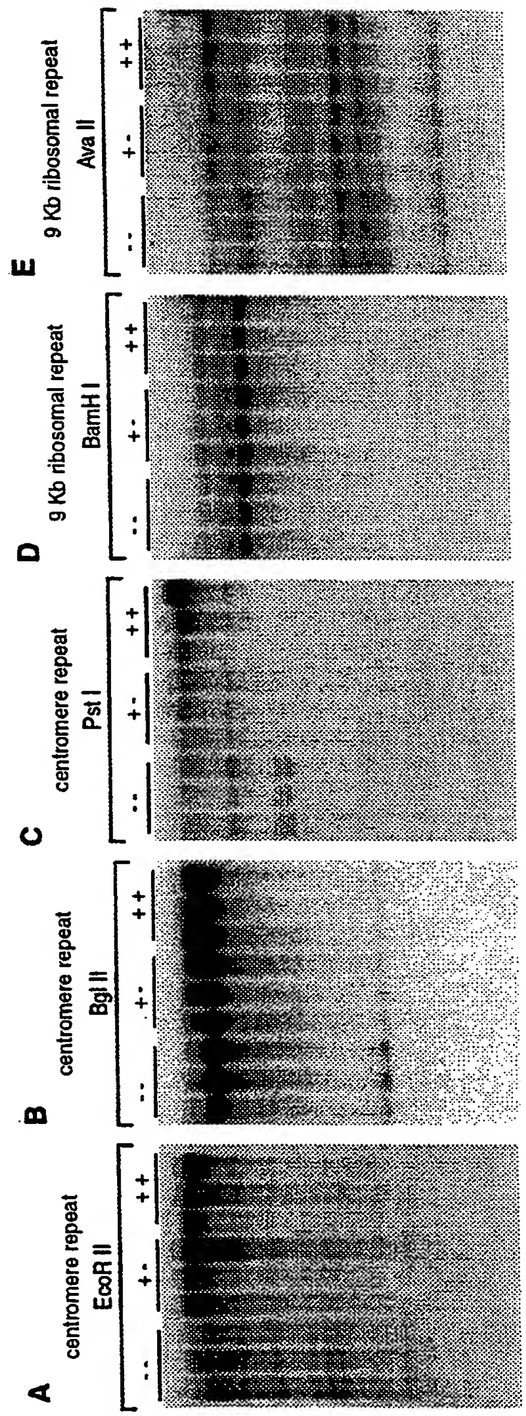
9 kb ribosomal repeat
HpaII
MspI
- -
+ -
+ +
- -
+ -
+ +
10 B
5s ribosomal repeat
HpaII
MspI
- -
+ -
+ +
- -
+ -
+ +
10 C
centromere repeat
HpaII
MspI
- -
+ -
+ +
- -
+ -
+ +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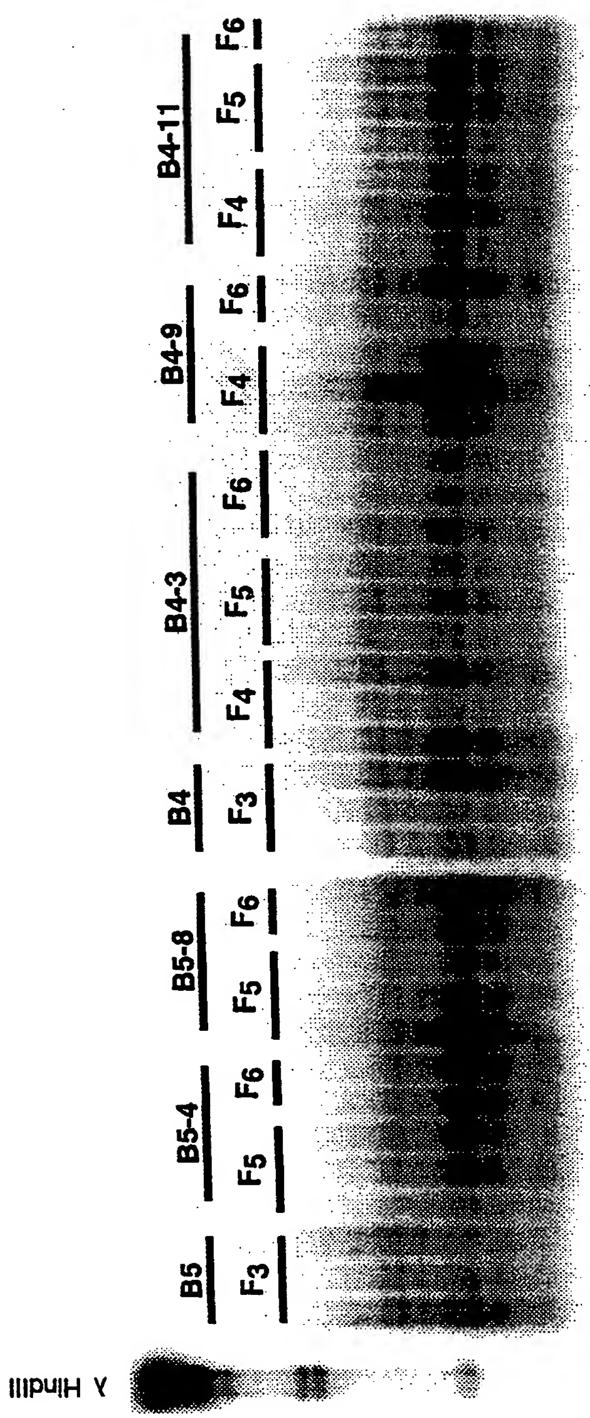
EcoR II
- - + - + +
B
centromere repeat
Bgl II
- - + - + +
C
centromere repeat
Pst I
- - + - + +
D
9 Kb ribosomal repeat
BamH I
- - + - + +
E
9 Kb ribosomal repeat
Ava II
- - + - + +

FIG. 11

FIG. 12

GENOTYPE	NUMBER OF PLANTS	TOTAL 5mCytosine (%)	% WT levels	% decrease
wild type	3	34.40 ± 0.55	100	0.0
heterozygous zmet2a-mu1	7	32.00 ± 0.90	93.0	7.0
homozygous zmet2a-mu1	5	30.40 ± 0.19	88.4	11.6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F3
B5-4
F5
F6
B5-8
F5
F6
B4
F3
B4-3
F4
F5
F6
B4-9
F4
F6
B4-11
F4
F5
F6
λ HindIII

FIG. 13

FIG. 14

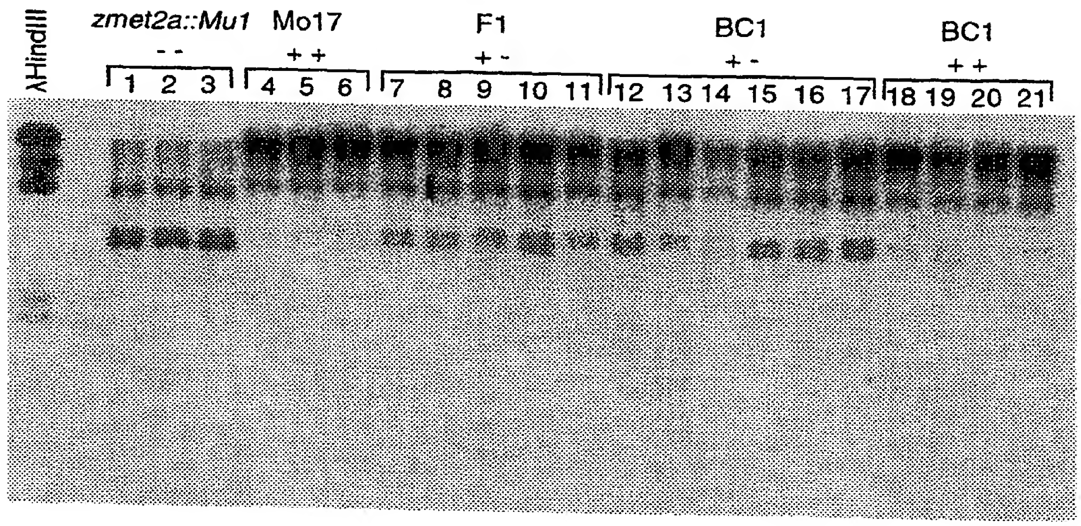
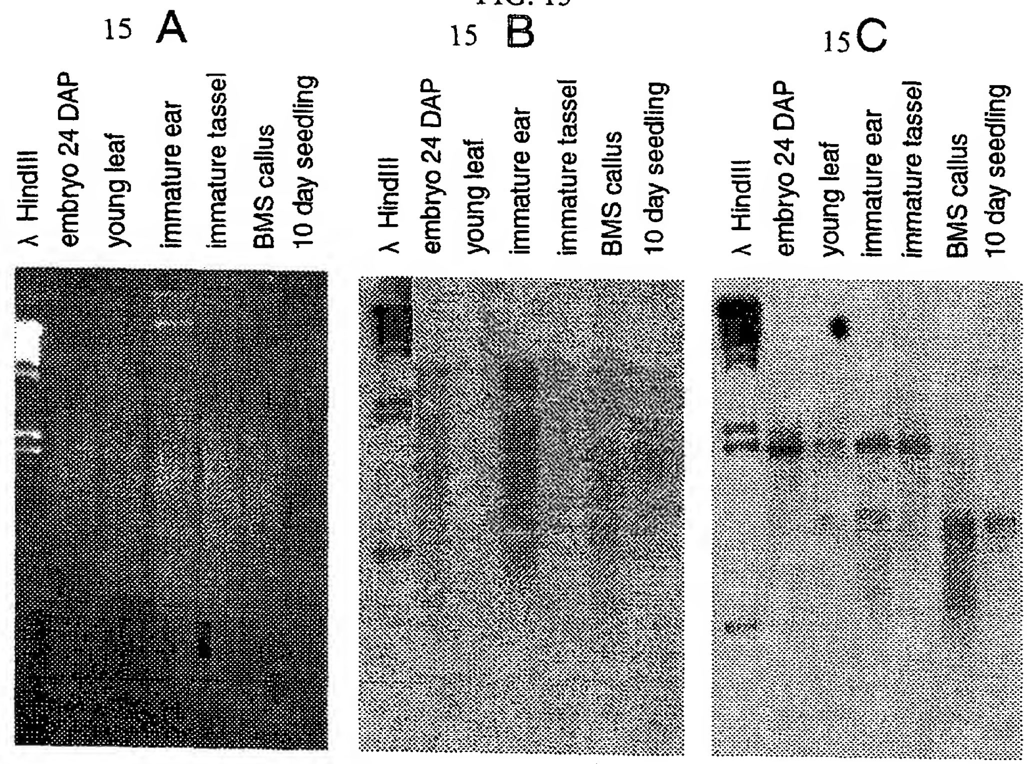

λHindIII
zmet2a::Mu1
- -
1 2 3
Mo17
+ +
4 5 6
F1
+ -
7 8 9 10 11
BC1
+ -
12 13 14 15 16 17
BC1
+ +
18 19 20 21

FIG. 15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λ HindIII
embryo 24 DAP
young leaf
immature ear
immature tassel
BMS callus
10 day seedling
15 B
λ HindIII
embryo 24 DAP
young leaf
immature ear
immature tassel
BMS callus
10 day seedling
15C
λ HindIII
embryo 24 DAP
young leaf
immature ear
immature tassel
BMS callus
10 day seedling

FIG.16

5' LTR

catgc**TGT**TGGGCCATGTGTCTAGTGTTGGCCCATTAACGTGTACA
CATATACTAGAAGTGTGTGTGGTGTAGAGAGAGTGCTGTATGTTTT
CCACATTCCAGAAAAATCC**ACA**TGGTATCAGAGCCAGG

PBS

3' LTR

PPT

GAGGGGGAG**TGT**TGGGCCATGTGTCTAGTGTTGGCCCATTAACGTG
TACACATATACTAGAAGTGTGTGTGGTGTAGAGAGAGTGCTGTATG
TTTTCCACATTCCAGAAAAATCC**ACA**catgc

FIG. 17

	Gag	Protease
SPRITE-1 -	CYNCGNVGHIARNC	TQVTQLKWILDSGASKH
hopscotch -	CQVCSRVGHTALNC	QNGSNVPWYTDTGATDH
retrofit -	CQVCFKRGHTAADC	SYGIDTNWYIDTGATDH
arabpolprt-	CSNCGRTGHEKKEC	GKTKLGDIILDSGASHH
copia -	CHHCGREGHIKKDC	SVMDNCGFVLDSGASDH

Integrase

SPRITE-1 -	QVKILRPDN-GTEYVNKGFNAFLSRNGILHQTSCPDTPPQNGVAERKNRHILE
hopscotch -	KIIAFQSDW-GGE--YEKLNAHFKTIGIHHQVSCPHTHQQNGAAERKHRHIVE
retrofit -	KIIAMQTDWRGGR--YQKLNSFFAQIGLIIMCHVLTLIRQNGSAERKHRHIVE
arabpolprt-	TVKMVRSDN-GTE--FMCLSSYFRENGIIHQTSCVGTPQQNGRVERKHRHILN
copia -	KVVYLYIDN-GREYLSNEMRQFCVKKGISYHLTVPHTPQLNGVSERMIRTITE

Reverse Transcriptase

SPRITE-1 -	RYKARLVARGYSQTYGIDYDETFAPVAKMSTVRTLISCAANFGWPLYQLDVKNAFLHGDLQEEVYMEIPPG(59)AILAVYVDDIII
hopscotch -	RLKARLVAKGFKQQYGIDYDDTFSPVVKHSTIRLVLSLAVSQKWSLRQLDVQNAFLHGILEETVYMKQPPG(59)IYILVYVDDIII
Retrofit -	RYKARLVAKGFKQRYGIDYEDTFSPVVKAATIRIILSIAVSRGWSLRQLDVQNAFLHGFLEEEVYMQQPPG(59)MFVLVYVDDIIV
Arabpolprt-	RYKARLVVQGNKQVEGEDYKETFAPVVRMTTVRTLLRNVAANQWEVYQMDVHNAFLHGDLEEEVYMKLPPG(59)LRVLIYVDDLLI
copia -	RYKARLVARGFTQKYQIDYEETFAPVARISSFRFILSLVIQYNLKVHQMDVKTAFLNGTLKEEIYMRLPQG(59)IYVLLYVDDVVI

RNase H

SPRITE-1 -	DADWGSCLDDRRSTSGYCVFVGG-NLVSWRSKKQSVVSRSTAEAEYRAMALAICEMLWIKGLL(25)NPVQHDRTKHVEIDRFF
hopscotch -	DADWAGCPDDRKSTGGYALFLGP-NLISWNSKKQSTVSRSSTEAEYKAMANATAEVIWLQSLL(25)KPIFNARTKHIEVDFHF
retrofit -	DADWAGSIDDRKSTGGFAVFLGS-NLVSWSARKQPTVSRSSTEAEYKAVANTTAELIWVQTLL(25)NPVFHARTKHIEVDYHF
arabpolprt-	DSDWQSCPLTRRSISAYVVLLGG-SPISWKTKKQDTVSHSSAEAEYRAMSYALKEIKWLRKLL(25)NPVFHERTKHIESDCHS
copia -	DSDWAGSEIDRKSTTGYLFKMFDFNLICWNTKRQNSVAASSTEAEYMALFEACREALWLKFLL(25)NPSCHKRAKHIDIKYHF

FIG. 18

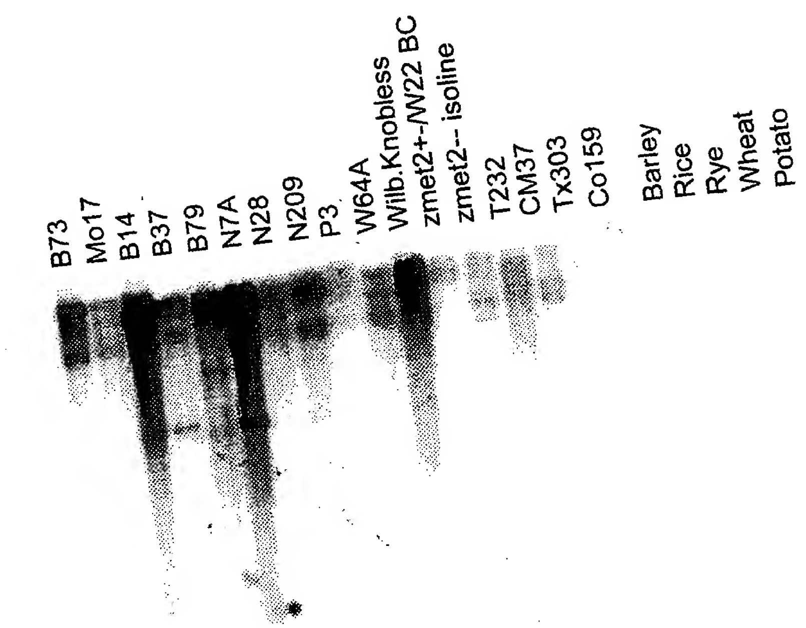
B73
Mo17
B14
B37
B79
N7A
N28
N209
P3
W64A
Wilb.Knobless
zmet2+-/W22 BC
zmet2-- isoline
T232
CM37
Tx303
Co159
Barley
Rice
Rye
Wheat
Potato

FIG. 19

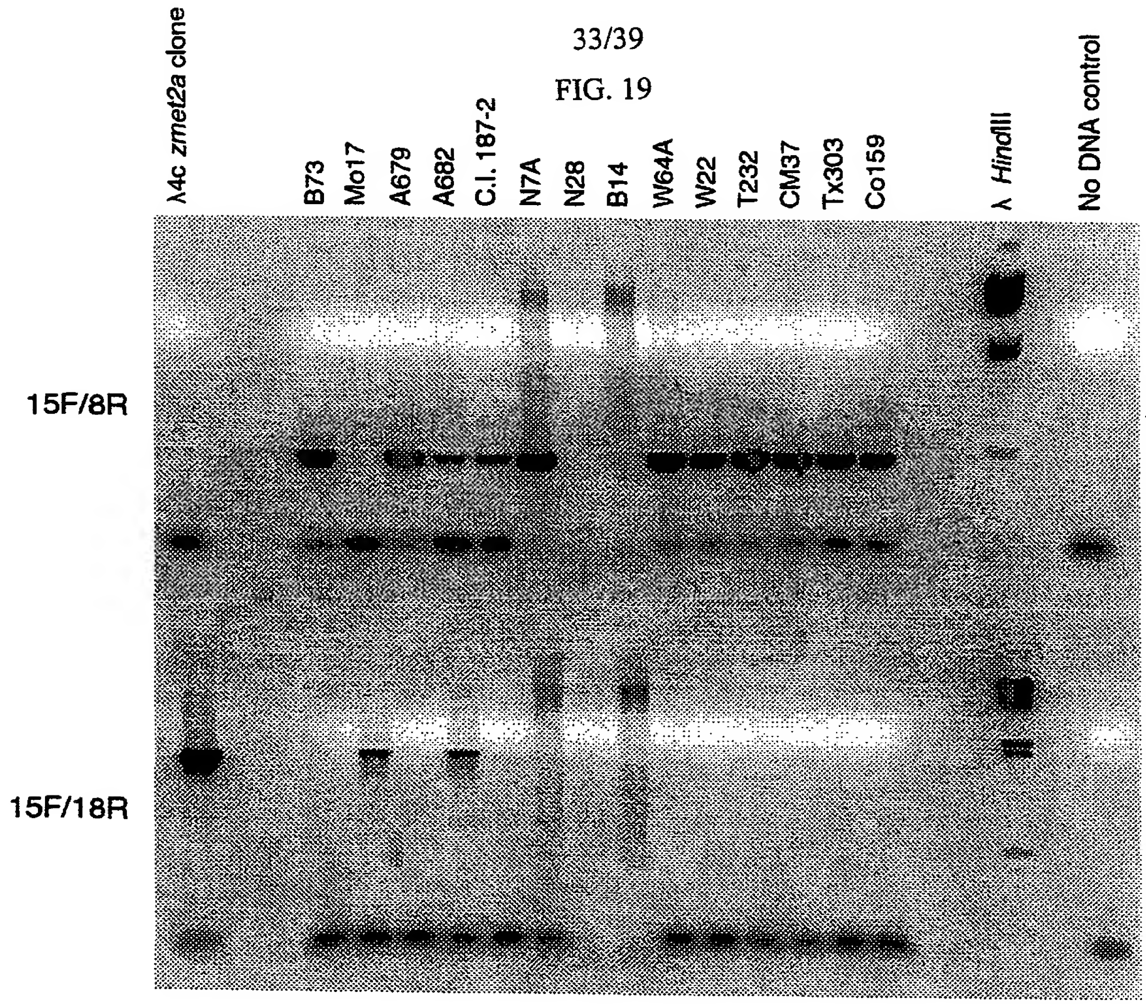
λ4c zmet2a clone
B73
Mo17
A679
A682
C.I. 187-2
N7A
N28
B14
W64A
W22
T232
CM37
Tx303
Co159
λ HindIII
No DNA control
15F/8R
15F/18R

FIG. 20

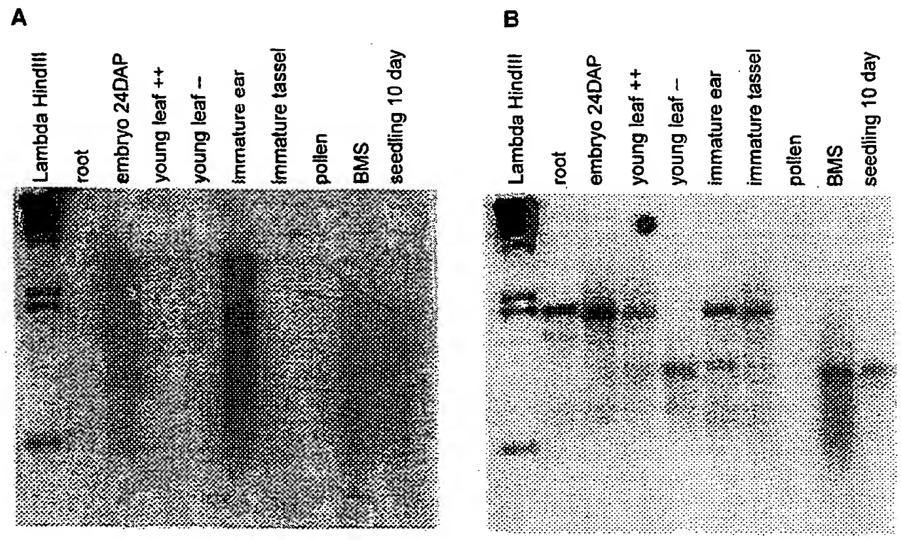
A
Lambda HindIII
root
embryo 24DAP
young leaf ++
young leaf --
immature ear
immature tassel
pollen
BMS
seedling 10 day
B
Lambda HindIII
root
embryo 24DAP
young leaf ++
young leaf --
immature ear
immature tassel
pollen
BMS
seedling 10 day

FIG. 21

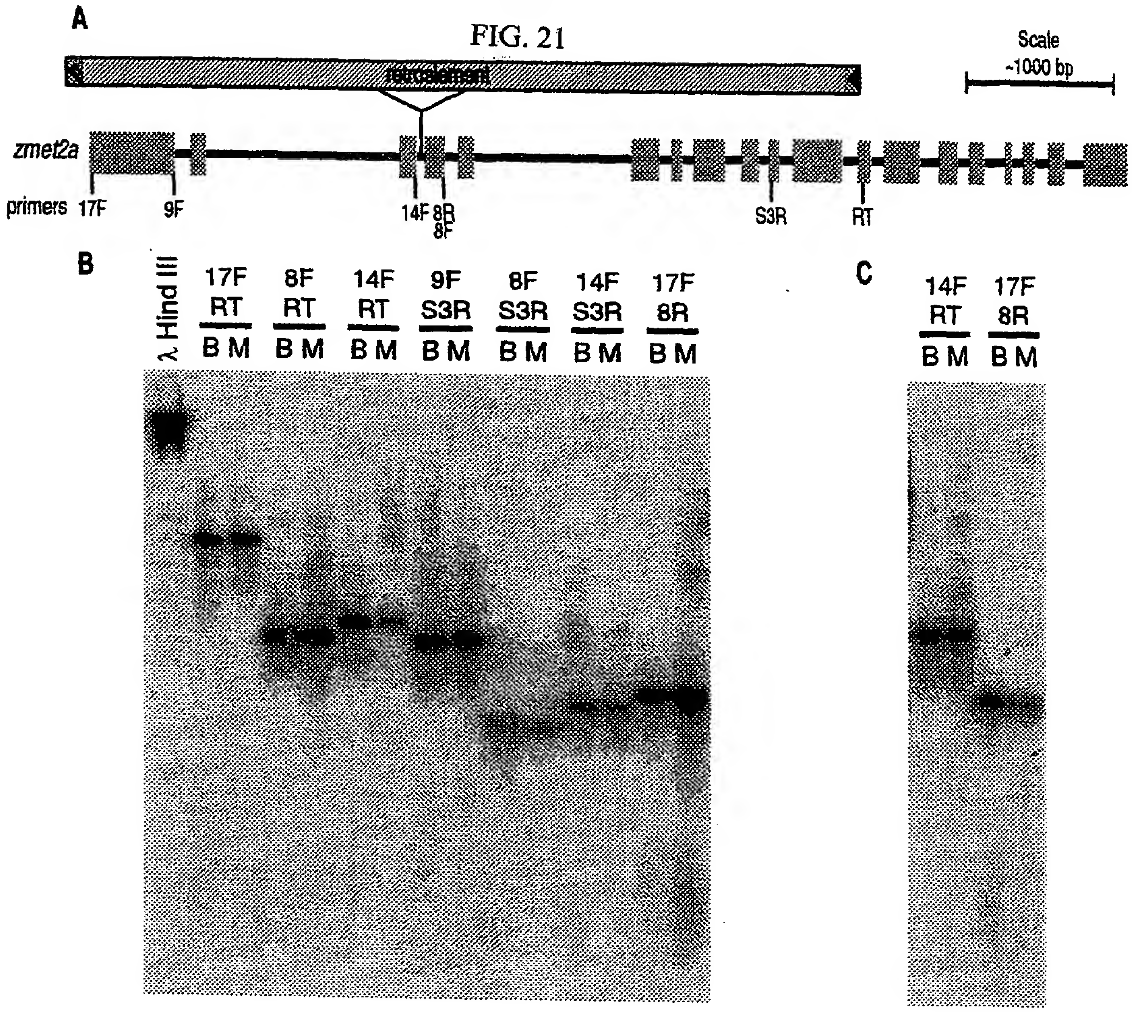
A
Scale
~1000 bp
retroelement
zmet2a
primers 17F 9F 14F 8R 8F S3R RT
B
λ Hind III
17F RT
8F RT
14F RT
9F S3R
8F S3R
14F S3R
17F 8R
B M B M B M B M B M B M B M
C
14F RT
17F 8R
B M B M

FIG. 22

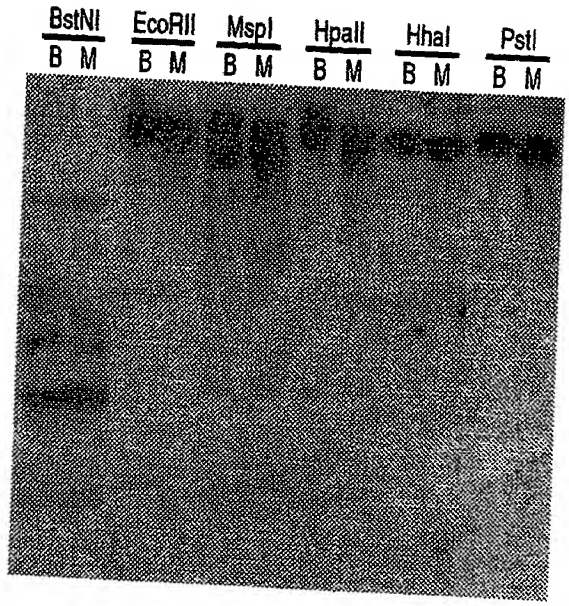

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FIG. 23

GGGAATTCGATTACTCACTATAGCGCTCGAGCGGCCGCCCGGGCAGGTTCGAAAACCATC
AACCTAACGATGTAATGGAGTATGGTGGTTCCCCCAAGACAGAGTTCCAGCGCTACATTC
GACTTGGTCGTAAAGACATGTTGGATTGGTCGTTTGGTGAGGAGGCTGGTCCAGATGAAG
GCAAGCTCTTGGATCACCAGCCCTTACGGCTTAACAATGATGATTATGAGCGGGTTAAGC
AAATTCCTGTCAAGAAGGGAGCCAACTTCCGTGACCTAAAGGGTGTCAAGGTTGGAGCAA
ATAATGTTGTTGAGTGGGATCCAGAAGTCGAACGTGTGTACCTTTCGTCTGGGAAACCAC
TGGTTCCTGACTATGCGATGTCATTCATCAAGGGCAAATCACTCAAGCCATTCGGGCGCC
TGTGGTGGGACCAGACGGTTcCTACAGTTGTGACCAGAGCAGAGCCTCATAACCAGGTTAT
ATTGCATCCGACTCAAGCAAGAGTCTTGACTATCCGGGAGAACGCAAGGTTACAGGGCTT
CCCCGATTACTACCGATTGTTTGGACCGATCAAGGAGAAGTATATTCAAGTCGGGAACGC
AGTGGCAGTCCCTGTTGCACGGGCACTGGGCTACTGTCTGGGTCAAGCCTACCTGGGTGA
ATCTGACGGGAGTCAGCCTCTGTACCAGCTGCCTGCAAGTTTTACCTCTGTGGGGCGAAC
CGCGGTTCAGGCGAATGCCGCTTCTGTTGGCACTCCTGCGGGGGAGGTAGTCGAGCAGTA
AAAGGATAGCGGAGCAACCCTGGTTGGTATTTTGATTCGAGCCCATCCAGTAGCATGTTT
ACCAATAAATAATCATTGGTCGTGCTGATTCTTATGGTTGGAGATGAATGTATGTAGGGT
GTACTCGAGCTCGAGTGCTTGTTGTACTGTAGGTTGAGGTTTCTCATCCATTGGCCTGCC
TATTTGTGGATGACGTTTCATTTCAGATTAGCAATGTGCTTATTTAAGGTTTCGTCATGT
ACCTGTATTCTACAATCCACTATTGTTTCCAAAGACAGCATTTGATCCTTAAAAAAAACT
GTAAAAAAAAAAAAACAGTGCCCGAAAAGCCGCAAAAAAAAAAAAAAAAAAAACCTGCCC
GGGCGGCCGCTCGAGCCCTATAGTGAGTAATCGAATTCCC

FIG. 24

EFDYSL*RSSGRPGRFENHQPNDVMEYGGSPKTEFQRYIRLGRKDMLDWS
FGEEAGPDEGKLLDHQPLRLNNDDYERVKQIPVKKGANFRDLKGVKVGAN
NVVEWDPEVERVYLSSGKPLVPDYAMSFIKGKSLKPFGRLWWDQTVPTVV
TRAEPHNQVILHPTQARVLTIRENARLQGFPDYYRLFGPIKEKYIQVGNA
VAVPVARALGYCLGQAYLGESDGSQPLYQLPASFTSVGRTAVQANAASVG
TPAGEVVEQ*

FIG. 25

```
667  KVQNHQPNDVMEYGGSPKTEFQRYIRLSRKDMLDWSFGEGAGPDEGKLLDHQPLRLNNDD  726
     +  +NHQPNDVMEYGGSPKTEFQRYIRL RKDMLDWSFGE AGPDEGKLLDHQPLRLNNDD
15   RFENHQPNDVMEYGGSPKTEFQRYIRLGRKDMLDWSFGEEAGPDEGKLLDHQPLRLNNDD  74

727  YERVQQIPVKKGANFRDLKGVRVGANNIVEWDPEIERVKLSSGKPLVPDYAMSFIKGKSL  786
     YERV+QIPVKKGANFRDLKGV+VGANN+VEWDPE+ERV  LSSGKPLVPDYAMSFIKGKSL
75   YERVKQIPVKKGANFRDLKGVKVGANNVVEWDPEVERVYLSSGKPLVPDYAMSFIKGKSL  134

787  KPFGRLWWDETVPTVVTRAEPHNQVIIHPTQARVLTIRENARLQGFPDYYRLFGPIKEKY  846
     KPFGRLWWD+TVPTVVTRAEPHNQVI+HPTQARVLTIRENARLQGFPDYYRLFGPIKEKY
135  KPFGRLWWDQTVPTVVTRAEPHNQVILHPTQARVLTIRENARLQGFPDYYRLFGPIKEKY  194

847  IQVGNAVAVPVARALGYCLGQAYLGESEGSDPLYQLPPSFTSVGGRTAGQARASPVGTPA  906
     IQVGNAVAVPVARALGYCLGQAYLGES+GS PLYQLP SFTSV GRTA QA A+ VGTPA
195  IQVGNAVAVPVARALGYCLGQAYLGESDGSQPLYQLPASFTSV-GRTAVQANAASVGTPA  253

907  GEVVEQ 912
     GEVVEQ
254  GEVVEQ 259
```

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/06456

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/10 C12N15/63 C12N5/14 C12N15/83 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Olhoft P.M.: "Cloning and characterization of the 5-methylcytosine methyltransferase gene in maize (zea mays) plants and tissue cultures" UNIV. OF MINNESOTA (0130) Degree: PHD Date:1998 pp:137 ABSTR. INT. B 1999, 59 (9),4638;Avail.: UMI,Order No. DA9907518 XP000900933	1-33
A	-& OLHOFT P.M. ET AL.: "Zea mays DNA (cytosine-5)-methyltransferase gene, complete sequence" EMBL DATABASE ENTRY T01661; ACCESSION NO. T01661, 19 February 1999 (1999-02-19), XP002146224	1-33

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2000	25/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schönwasser, D

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	CAO X. ET AL.: "Conserved plant genes with similarity to mammalian de novo DNA methyltransferase" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 97, no. 9, 25 April 2000 (2000-04-25), page 4979-4984 XP002146225 figure 3	1-33
A	HENIKOFF S. ET AL.: "A DNA methyltransferase homolog with a chromodomain exists in multiple polymorphic forms in Arabidopsis" GENETICS, vol. 149, no. 1, May 1998 (1998-05), pages 307-318, XP002146226 the whole document	1-18
P,A	WALBOT V.: "Maize ESTs from various cDNA libraries sequences at Stanford University; 687002G02.y1 687 - Early embryo from Delaware Zea mays cDNA, mRNA sequence." EMBL DATABASE ENTRY AW065905; ACCESSION NO.:AW065905, 18 October 1999 (1999-10-18), XP002146227	1-18
P,A	WALBOT V.: "Maize ESTs from various cDNA libraries sequences at Stanford University;707027A05.x2 707 - Mixed adult tissues from Walbot lab (SK) Zea mays cDNA, mRNA sequence." EMBL DATABASE ENTRY AW330561; ACCESSION NO. AW330561, 1 February 2000 (2000-02-01), XP002146228	1,19-33

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only	
International Application No.	09/914001
International Filing Date	
Name of receiving Office and "PCT International Application"	

Applicant's or agent's file reference
(if desired) (12 characters maximum)

Box No. I TITLE OF INVENTION

Nucleic Acid and Amino Acid Sequences Encoding Class II DNA Methyltransferases

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Wisconsin Alumni Research Foundation
P.O. Box 7365
Madison, Wisconsin 53707-7365

☐ This person is also inventor.

Telephone No.
608-263-2500

Facsimile No.
608-263-1064

Teleprinter No.

State *(that is, country)* of nationality:
United States of America

State *(that is, country)* of residence:
United States of Americ

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Pioneer Hi-Bred, International, Inc.
400 Locust Street
Suite 800
P.O. Box 800
Des Moines, Iowa 50306-3453

This person is:

☒ applicant only

☐ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:
United States of America

State *(that is, country)* of residence:
United States of America

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Mueller, Lisa V.
ROCKEY, MILNAMOW & KATZ, LTD.
180 North Stetson Avenue
Two Prudential Plaza, Suite 4700
Chicago, Illinois 60601
U.S.A.

Telephone No.
312-616-5400

Facsimile No.
312-616-5460

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is:
Regents of The University of Minnesota 600 University Gate Way 200 Oak Street S.E. Minneapolis, MN 55455	☒ applicant only ☐ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality: United States of America	State *(that is, country)* of residence: United States of America

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is:
Kaeppler, Shawn M. 5290 County Highway A Oregon, Wisconsin 53575	☐ applicant only ☒ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality: United States of America	State *(that is, country)* of residence: United States of America

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is:
Springer, Nathan Michael 918 Washington Street Northfield, MN 55057	☐ applicant only ☒ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality: United States of America	State *(that is, country)* of residence: United States of America

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is:
Muszynski, Michael Gerard 5505 Shriver Avenue #2 Johnston, IA 50131	☐ applicant only ☒ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality: United States of America	State *(that is, country)* of residence: United States of America

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Continuation of Box N . III FURTHER APPLICANTS AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Papa, Charles Marvin
903 Beacon Street #1
Madison, Wisconsin 53715

This person is:

- [ ] applicant only
- [x] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: United States of America

State *(that is, country)* of residence: United States of America

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [x] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4 9(a) *(mark the applicable check-boxes, at least one must be marked)*

Regional Patent

☒ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **TZ** United Republic of Tanzania, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* ....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*

☒ **AE** United Arab Emirates
☒ **AL** Albania
☒ **AM** Armenia
☒ **AT** Austria
☒ **AU** Australia
☒ **AZ** Azerbaijan
☒ **BA** Bosnia and Herzegovina
☒ **BB** Barbados
☒ **BG** Bulgaria
☒ **BR** Brazil
☒ **BY** Belarus
☒ **CA** Canada
☒ **CH and LI** Switzerland and Liechtenstein
☒ **CN** China
☒ **CR** Costa Rica
☒ **CU** Cuba
☒ **CZ** Czech Republic
☒ **DE** Germany
☒ **DK** Denmark
☒ **DM** Dominica
☒ **EE** Estonia
☒ **ES** Spain
☒ **FI** Finland
☒ **GB** United Kingdom
☒ **GD** Grenada
☒ **GE** Georgia
☒ **GH** Ghana
☒ **GM** Gambia
☒ **HR** Croatia
☒ **HU** Hungary
☒ **ID** Indonesia
☒ **IL** Israel
☒ **IN** India
☒ **IS** Iceland
☒ **JP** Japan
☒ **KE** Kenya
☒ **KG** Kyrgyzstan
☒ **KP** Democratic People's Republic of Korea
☒ **KR** Republic of Korea
☒ **KZ** Kazakhstan
☒ **LC** Saint Lucia
☒ **LK** Sri Lanka
☒ **LR** Liberia
☒ **LS** Lesotho
☒ **LT** Lithuania
☒ **LU** Luxembourg
☒ **LV** Latvia
☒ **MA** Morocco
☒ **MD** Republic of Moldova
☒ **MG** Madagascar
☒ **MK** The former Yugoslav Republic of Macedonia
☒ **MN** Mongolia
☒ **MW** Malawi
☒ **MX** Mexico
☒ **NO** Norway
☒ **NZ** New Zealand
☒ **PL** Poland
☒ **PT** Portugal
☒ **RO** Romania
☒ **RU** Russian Federation
☒ **SD** Sudan
☒ **SE** Sweden
☒ **SG** Singapore
☒ **SI** Slovenia
☒ **SK** Slovakia
☒ **SL** Sierra Leone
☒ **TJ** Tajikistan
☒ **TM** Turkmenistan
☒ **TR** Turkey
☒ **TT** Trinidad and Tobago
☒ **TZ** United Republic of Tanzania
☒ **UA** Ukraine
☒ **UG** Uganda
☒ **US** United States of America continuation-in-part
☒ **UZ** Uzbekistan
☒ **VN** Viet Nam
☒ **YU** Yugoslavia
☒ **ZA** South Africa
☒ **ZW** Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet

☐
☐

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4 9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months [illegible] to be regarded as withdrawn [illegible] the applicant [illegible]

Form PCT RO 101 (second sheet) (January 2000) *See Notes to the request form*

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:** in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication **"patent of addition,"** or **"certificate of addition,"** or if, in Box No. V., the name of the United States of America is accompanied by an indication **"continuation"** or **"continuation-in-part"**: in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed:** in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, the **earlier application is an ARIPO application:** in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudical disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation of Box IV

Chapa, Lawrence
Elliott, Thomas C.
Erickson, Randall
Geimer, Steve D.
Hoover, Allen J.
Katz, Martin L.
Lyons, Kathleen A.
Milnamow, John P.
Odell, Paul M.
Polit, Robert B.
Ramesh, Elaine M.
Rockey, Keith V.
Rollin, John
Ross, Thomas I.
Scott, Ted R.
Siegel, Joel
Vargo, Paul V.

Continuatin of Box V

United Stat s of America - continuation-in-part of U.S. Serial No. 60/169,858 which is a continuation-in-part of U.S. Serial No. 60/123,888

Box No. VI PRIORITY CLAIM		☐ Further priority claims are indicated in the Supplemental Box.		
Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country	regional application:* regional Office	international application: receiving Office
item (1) 11 MARCH 1999 (11/03/99)	60/123,888	U.S.		
item (2) 09 DECEMBER 1999 (09/12/99)	60/169,858	U.S.		
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): 1 and 2

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA/ EP

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country (or regional Office)

Box No. VIII CHECK LIST: LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	6
description (excluding sequence listing part)	:	57
claims	:	5
abstract	:	1
drawings	:	40
sequence listing part of description	:	
Total number of sheets	:	109

This international application is **accompanied** by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☒ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as items(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify):*

Figure of the drawings which should accompany the abstract: 1B

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Mueller, Lisa V., Reg. No. 38,978

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA/
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference **WIS49870051PCT**

Applicant
Wisconsin Alumni Research Foundation, et al.

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE .... **240.00** T

2. SEARCH FEE .... **990.00** S

International search to be carried out by **EP**

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains **64** sheets.

first 30 sheets .... **427.00** b_1

34 (remaining sheets) x **$10.00** (additional amount) = **340.00** b_2

Add amounts entered at b_1 and b_2 and enter total at B . . **767.00** B

Designation Fees

The international application contains **82** designations.

8 (number of designation fees payable *(maximum 10)*) x **92.00** (amount of designation fee) = **736.00** D

Add amounts entered at B and D and enter total at I .... **1,503.00** I

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* .... **30.00** P

5. TOTAL FEES PAYABLE

Add amounts entered at T, S, I and P, and enter total in the TOTAL box **2,763.00** TOTAL

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

- ☐ authorization to charge deposit account (see below)
- ☒ cheque
- ☐ postal money order
- ☐ bank draft
- ☐ cash
- ☐ revenue stamps
- ☐ coupons
- ☐ other *(specify)*:

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ **US**

☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

04-1644	**10/03/00**	
Deposit Account Number	Date *(day/month/year)*	Signature

Form PCT/RO/101 (Annex) (January 1999) LegalStar 1999, Form PCTRFEE *See Notes to the fee calculation sheet*

PCT

GENERAL POWER OF ATTORNEY

(for several International applications filed under the Patent Cooperation Treaty)
(PCT Rule 90.5)

The undersigned person(s):
(Family name followed by given name; for a full legal entity, full official designation. The address must include postal code and name of country.)

Regents of The University Of Minnesota
600 University Gate Way
200 Oak Street S.E.
Minneapolis, MN 55455

hereby appoint(s) the following person as: ☒ agent ☐ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Chapa, Lawrence J.	Reg. No. 39,135	Milnamow, John P.	Reg. No. 20,635	Rockey, Keith V.	Reg. No. 24,713
Erickson, Randall T.	Reg. No. 33,872	Mueller, Lisa V.	Reg. No. 38,978	Rollins, John F.	Reg. No. 38,013
Geimer, Stephen D.	Reg. No. 28,846	Odell, Paul M.	Reg. No. 28,332	Ross, Thomas I	Reg. No. 29,275
Hoover, Allen J.	Reg. No. 24,103	Polit, Robert B.	Reg. No. 33,993	Siegel, Joel E.	Reg. No. 25,440
Katz, Martin L.	Reg. No. 25,011	Ramesh, Elaine M.	Reg. No. 43,032	Vargo, Paul M.	Reg. No. 29,116
Lyons, Kathleen A.	Reg. No. 31,852				

of the firm **Rockey, Milnamow & Katz, Ltd., 180 North Stetson Avenue, Suite 4700, Chicago, Illinois 60601 U.S.A.**, to represent the undersigned before:

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with any and all international applications filed by the undersigned with the following Office United States and to make or receive payments on behalf of the undersigned.

Signature(s) *(where there are several persons, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading this power):*

____
Name

____
Title

Date:____________________